FORM PTO-1390 (REV. 9-2001)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY 'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		43550
		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) **10/088138**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/02540	14 September 2000	17 September 1999

TITLE OF INVENTION
Novel Animal Model of Alzheimer Disease with Amyloid Plaques and Mitochondrial Dysfunctions

APPLICANT(S) FOR DO/EO/US   Saliha Moussaoui-Mrabet, Veronique Blanchard-Bregeon, Assunta Imperato, Bruno Bonici, Gunter Tremp, Christian Czech

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☑ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.

4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).

5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
    a. ☐ is attached hereto (required only if not communicated by the International Bureau).
    b. ☑ has been communicated by the International Bureau.
    c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☑ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
    a. ☑ is attached hereto.
    b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).

7. ☐ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
    a. ☐ are attached hereto (required only if not communicated by the International Bureau).
    b. ☐ have been communicated by the International Bureau.
    c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
    d. ☐ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☑ A FIRST preliminary amendment.

14. ☐ A SECOND or SUBSEQUENT preliminary amendment.

15. ☐ A substitute specification.

16. ☐ A change of power of attorney and/or address letter.

17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.

18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).

19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

20. ☑ Other items or information: Search Report

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/088138	INTERNATIONAL APPLICATION NO. ПХТ/ФР00/02540	ATTORNEY'S DOCKET NUMBER 43550

	CALCULATIONS PTO USE ONLY

21. ☑ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☑ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	54 - 20 =	34	x **$18.00**	$ 612.00	
Independent claims	1 - 3 =	0	x **$84.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$ 280.00	

TOTAL OF ABOVE CALCULATIONS =	$ 1912.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +	$	
SUBTOTAL =	$ 1912.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$	
TOTAL NATIONAL FEE =	$ 1912.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$ 1912.00	
	Amount to be refunded:	$
	charged:	$

a. ☑ A check in the amount of $ _____1912.00_____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ____18-2220____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. __18-2220__ . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

_____
SIGNATURE

Dean H. Nakamura
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NAME

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REGISTRATION NUMBER

Initial Information Data Sheet

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Application Information

Title Line One::	Novel Animal Mode of Alzheimer Disease
Title Line Two::	with Amyloid Plaques and Mitrochondrial
Title Line Three::	Dysfunction
Total Drawing Sheets::	27
Formal Drawings?::	No
Application Type::	Utility
Docket Number::	43550

Representative Information

Registration Number::	33,981

43550   <u>PATENT</u>

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of   :

Moussaoui-Mrabet et al.   :   Patent Art Unit: TBA

Serial No. Based on PCT/FR00/02540   :   Examiner: TBA

Filed: 15 March 2002   :
                                              :
For: Novel Animal Model of Alzheimer   :
        disease with Amyloid Plaques and   :
        Mitochondrial Dysfunctions   :

<u>PRELIMINARY AMENDMENT</u>

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to prosecution on the merits, kindly amend the application as follows.

IN THE CLAIMS:

Kindly amend the claims as follows.

10. (Amended) A method for identifying compounds which can be used for treating neurodegenerative diseases comprising exposing said compounds to the animal model of any one of claims 1 to 9.

11. (Amended) Cell which is extracted from an animal model as described in accordance with any one of claims 1 to 9.

PRELIMINARY AMENDMENT
Serial No. Based on PCT/FR00/02540
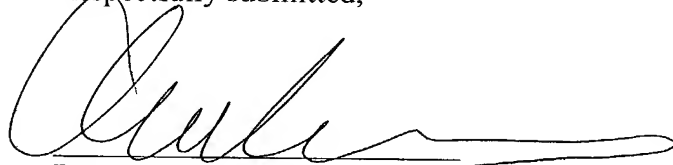
12. (Amended) A method for identifying compounds which can be used for treating neurodegenerative diseases comprising exposing said compounds to the cell of claim 11.

Kindly add the following new claims.

13. The method of claim 10, wherein said neurodegenerative disease is Alzheimer's disease.

14. The method of claim 12, wherein said neurodegenerative disease is Alzheimer's disease.

Favorable consideration and early indication of allowance are solicited earnestly.

Respectfully submitted,

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Dated: 15 March 2002

8.   Animal model according to Claim 7, characterized in that the proteins are intramitochondrial proteins.

9.   Model according to Claim 8,
5   characterized in that the proteins are the BAX and/or cytochrome C proteins.

10.   Use of the animal model as described in accordance with Claims 1 to 9 ~~A method~~ for identifying compounds which can be used for treating neurodegenerative
10   diseases preferably Alzheimer's disease. comprising exposing said compounds to the animal model of any one of claims 1 to 9

11.   Cell which is extracted from an animal model as described in accordance with Claims 1 to 9.

12.   Use of a cell as described in accordance ~~any one of~~
with Claim 11 ~~A method~~ for identifying compounds which can be
15   used for treating neurodegenerative diseases preferably Alzheimer's disease comprising exposing said compounds to the cell of claim 11.

NOVEL ANIMAL MODEL OF ALZHEIMER'S DISEASE

EXHIBITING BOTH AMYLOID PLAQUES AND

MITOCHONDRIAL DYSFUNCTION

The present invention relates to the field of

5   transgenic animal models and, more specifically, to

animal models of Alzheimer's disease. The invention

relates to a novel animal model of Alzheimer's disease

which exhibits both amyloid plaques and mitochondrial

dysfunction.

10   Alzheimer's disease (AD) is a progressive

neurodegenerative disease which affects a large

proportion of the elderly population. This disease is

characterized at the clinical level by a loss of memory

and a decline in cognitive functions and, on the neuro-

15 pathological level, by the presence in the brain of

intracellular neurofibrillary deposits and extra-

cellular deposits of the β-amyloid peptide (A-β) which

forms the amyloid plaques (Yanker et al., 1996). In

addition to these signs, there are a substantial number

20 of other abnormal changes, including impairments in the

immune and inflammatory systems, as well as an

impairment in mitochondrial function which can lead to

an increase in oxidative stress and activation of the

mechanisms of apoptosis, leading eventually to cell

25 death.

Amyloid plaques are in the main composed of

A-β peptides, having 40 or 42 residues, which are

generated during the proteolytic process of the
β-amyloid peptide precursor protein (APP). The
extracellular deposits of A-β are very specific for AD
and associated disorders. They are the early and

5   invariable feature of all the forms of AD, including
the familial forms (FAD). The FADs appear relatively
early on (between 40 and 60 years of age) and are due
to mutations in the APP gene in 5% of FAD cases (19
families) with six single or double false-sense

10 mutations; in the presenilin 1 (PS 1) gene in 50 to 70%
of FAD cases (> to 50 families), with more than 40
different mutations identified to date; and in the
presenilin 2 (PS 2) gene in fewer FAD cases, with 2
false-sense mutations described in 8 families (for

15 review, see Price and Sisodia, 1998). Mutations in
these three genes have been demonstrated to induce
changes in the proteolysis of APP, leading to
overproduction of A-β and to the early appearance of
pathology and symptoms which are similar to those of

20 the sporadic forms of AD.

        In addition to the mutations in the APP, PS1
and PS2 genes, other factors contributing to AD have
been demonstrated in man, in particular mitochondrial
dysfunction (Beal, 1998). Thus, different investigative

25 approaches in individuals suffering from AD
demonstrated that mitochondrial dysfunction was
important in the appearance of the disease.

Thus, in accordance with a first approach, deficiencies in mitochondrial cytochrome C oxidase (COX or complex IV of the mitochondrial respiratory chain) activity have been demonstrated in the brains of human

5   patients suffering from AD (Parker et al., 1989; Parker et al., 1994; Mutisya et al., 1994; Kish et al., 1992). Mutations of the mitochondrial COX genes have also been demonstrated in late forms of AD (Davis et al., 1996, 1997). Mitochondrial COX activity is decreased by 52%

10 in *cybrid* cells in which the mitochondria of platelets from patients suffering from AD are fused to the cell line SH-SY5Y, which is characterized by a depletion of its mitochondrial DNA. This decrease in mitochondrial activity results in an overproduction of free radicals

15 and an increase in the basal concentration of Ca^{2+} (Sheehan et al., 1997).

A second approach, which is just as important, demonstrated the important role which mitochondrial dysfunction plays in the cell death which

20 is induced by apoptosis in patients suffering from AD (Su et al., 1997; Mac Gibbon et al., 1997; Tortosa et al., 1998; Nagy et al., 1997), and in particular the important role played by Bax, which has been identified in AD patient lesions (Bax being a mitochondrial

25 protein of the Bcl-2 family which is known to induce cell death by opening the mitochondrial megachannel and inducing the mitochondria to release apoptotic

molecules, including cytochrome C, which is the
substrate of COX and which activates caspases, as has
been demonstrated in cultured cells, in the cytosol).
In actual fact, Bax is concentrated both in the axonal

5   structures surrounding the senile plaques (the location
of bax is correlated with that of the A-β deposits in
adjacent sections of the same brain at the level of the
hippocampus in patients suffering from AD), and in
neurons which are carrying early neurofibrillary

10   degenerations, indicating that Bax plays a role in the
formation of neurofibrillary degenerations in patients
suffering from AD (MacGibbon et al., 1997; Tortosa
et al., 1998; Nagy et al., 1997).

It is clearly shown in the literature that
15   the neurotoxic properties of A-β which have been
demonstrated *in vitro* are associated with the
production of oxygenated free radicals (Pappola et al.,
1998). However, this finding does not appear to solve
the question of whether the oxidative stress is due to

20   mitochondrial dysfunction or whether the oxidative
stress might appear in vivo in the brain in association
with the A-β deposits. This is because the findings, of
which there a large number in the literature, which
demonstrate and/or suggest that the neurotoxicity of

25   A-β is mediated by oxidative stress are in fact only
in vitro (and not *in vivo*) findings. Furthermore, the

in vivo toxicity of A-β has been called into question in many reports (Papolla et al., 1998).

While it is known that the A-β deposits are associated with mitochondrial dysfunction in human cerebral tissue, there has to date been no reported finding which demonstrates an association between the mitochondrial dysfunction and the A-β deposits in a transgenic animal model of AD. Two recent publications on transgenic animals expressing mutated APP (animals developed by Hsiao et al., 1996) reported an increase in superoxide dismutase (SOD) and hemo-oxygenase-1 (HO-1), and an increase in lipid peroxidation and hydroxynonenal (HNE) in association with A-β deposits (Smith et al., 1998; Papolla et al., 1998). However, all these markers are oxidative stress markers which are situated downstream of the mitochondrial dysfunction and are not specific for this dysfunction. Furthermore, some of these markers used in the previously described studies, i.e. SOD and HO-1, are not oxidizing agents which induce neuronal death but are, on the contrary, known as being antioxidants which are involved in the defence mechanisms which are brought into play in reducing oxidative damage and cell death.

As far as the presenilins are concerned, mutations of PS1 have been reported to induce apoptosis in cultured cells and *in vivo* in the brains of

transgenic mice expressing mutated PS1 (Chiu et al.,

1999). However, this does not show that the apoptosis

induced by the PS1 mutations is mediated by

mitochondrial dysfunction. This is because the

5 apoptosis can be either the consequence of

mitochondrial dysfunction or the consequence of other

mechanisms of cell death which are not necessarily

involved in the mitochondrial dysfunction: for example,

it has been shown that inhibition of the protease

10 activity of interleukin-converting enzyme 1-β (ICE)

only prevents Bax-induced changes which are downstream

of the mitochondria (degradation of DNA, for example)

but has no effect on mitochondrial changes such as the

loss of the mitochondrial membrane potential and the

15 production of free radicals (Xiang et al., 1996).

Recent in-vitro studies have demonstrated that the PS1

mutations are involved in the overproduction of free

radicals, in the impairment of calcium homeostasis and

in the loss of mitochondrial membrane potential (Begley

20 et al., 1999; Guo et al., 1996, 1997, 1998). However,

these studies do not show any association between the

mitochondrial dysfunction and the A-β deposits in

transgenic mice expressing mutated PS1 since the

transgenic mice used in these studies for preparing

25 synaptosomes do not develop A-β deposits. There is

therefore no evidence in the literature which shows an

association between mitochondrial dysfunction and A-β

deposits *in vivo* in the brains of transgenic mice which
are expressing mutated PS1 (Chui et al., 1999) or
mutated APP (Hsiao et al., 1996; Irizzary et al.,
1997a, 1997b; Johson-Wood et al., 1997), or else
5 coexpressing mutated APP and mutated PS1 (Borchelt
et al., 1997; Holcomb et al., 1998).

The present invention therefore results from
the demonstration, for the first time, that amyloid
deposits are associated with mitochondrial dysfunction
10 *in vivo* in the brains of transgenic animals.
Furthermore, the invention also results from a search
for a novel animal model of AD which is more
representative and which reproduces the neuropathology
which is encountered in man.

15 The invention firstly relates, therefore, to
a transgenic animal model of Alzheimer's disease which
exhibits both amyloid plaques and mitochondrial
dysfunction. Advantageously, it coexpresses the
β-amyloid peptide precursor (APP) and a presenilin,
20 preferably PS_1.

A transgenic animal is understood as being
any non-human animal whose genome has been modified.
The modification of the genome can result from an
alteration or modification of one or more genes by
25 knock-in or knock-out. This modification can be due to
the action of conventional mutagenic or altering agents

or else brought about by site-directed mutagenesis, as described in Materials and Methods.

The modification of the genome can also result from the insertion of (a) gene(s) or the replacement of (a) gene(s) in its (their) wild-type or mutated form.

The modifications of the genome are advantageously carried out on reproductive stem cells, advantageously on their pronuclei.

Within the context of the present invention, the animal model is advantageously a mammal. In particular, it can be a mouse, a rat or a rabbit which is obtained using the conventional techniques of transgenesis. By way of an example illustrating one of the procedures of transgenesis, mention may be made of the method of microinjecting an expression cassette which comprises the modified genes in the two fertilized pronuclei, as described in Materials and Methods.

In this respect, the animal model of the invention is obtained by injecting an expression cassette which comprises a nucleic acid. This nucleic acid is preferably a DNA which can be a genomic DNA (gDNA) or a complementary DNA (cDNA).

Within the context of the model of the invention, the DNA encodes any gene which is able to play a part in the process of establishing the AD.

Advantageously, the gene encoded by the DNA is involved in the mechanism of producing the A-β peptide in its amyloidogenic form.

In particular, the DNA encodes mutated forms
5 of APP and/or presenilins, in particular PS1, such that the cells of the animal model coexpress the two mutated proteins.

The mutations in the APP gene can be one of the various mutations which have to date been described
10 in the literature. Preferably, the mutations in the APP gene are selected from the "Swedish" (S), "London" (L) and "Dutch" (D) mutations, taken on their own or in combination.

These mutations are amply described in the
15 literature and are generally characterized by the following modifications:

Nature and position	Swedish mutation	Dutch mutation	London mutation
in relation to APP770	K 670 N and M 671 L	E 693 Q and/or A 692 G	V 717 I
in relation to APP751	K 651 N and M 652 L	E 674 Q and/or A 673 G	V 698 I
in relation to APP695	K 595 N and	E 618 Q and/or	V 642 I

	M 596 L	A 617 G	
in relation to the A-β peptide (A42)		E 22 Q and/or A 21 G	V 46 I

The London mutation also comprises all the substitutions other than with isoleucine which are located in position 717 with reference to APP770, such

5   as the V 717 G and V 717 F mutations.

It is understood that the APP which can be used within the context of the invention can be in different isoforms, in particular in the 695, 751 and 770 forms, or in a truncated form such as the APP99

10 isoform.

The mutations in the PS1 gene can be one of the 40 mutations described to date in the literature. Preferably, the mutations in the PS1 gene are selected from the M146L, A246E, C410Y, H163R, L226V, L235P, etc.

15 mutations, taken on their own or in combination.

The M146L mutation is preferred for preparing a model according to the invention.

Within the context of the model of the invention, the DNA is placed under the control of

20 sequences which enable it to be expressed, in particular transcription promoter sequences.

Promoter sequences which may be mentioned are, very particularly, the HMG promoter (Gautier

et al., 1989), and also the PDGF promoter (Sasahan

et al., 1991), the Thy-1 promoter (Lüthi et al.,) and

the Prion gene promoter (Scott et al., 1992).

According to a particularly favourable

5   embodiment of the invention, the animal model comprises

the APP gene possessing the S, D and L mutations, with

this gene being placed under the control of the PDGF

promoter, and the PS1 gene possessing the M146 L

mutation, with this gene being placed under the control

10   of the HMG promoter.

The animal model according to the invention

is very advantageous since it is a model which is very

representative of AD. This is because this model

develops amyloid plaques from the age of 6 months,

15   which makes the time required for breeding the animals

very short, and coexpresses mutated APP and PS1

proteins at levels which are clearly higher than the

endogenous levels; at least 3 to 5 times and 2 to 3

times the endogenous levels of APP and PS1,

20   respectively.

Thus, the results described in the examples

show that the transgenic mouse which is coexpressing

mutated APP and mutated PS1 develops a neuropathology

of the Alzheimer's disease type; that is, it exhibits

25   A-β deposits having a fibrillary conformation,

neurodegenerative changes of the abnormal axonal

structure type, and activation of central nervous

system cells of the inflammatory type such as

astrocytes.

Particularly favourably, this model exhibits

mitochondrial dysfunction, which has also been shown in

5   patients suffering from AD, in addition to the amyloid

plaques.

The results described in the examples show

the involvement of mitochondrial dysfunction in the

neuropathology of these transgenic AD mice. In relation

10 to the findings which have been published to date in

the mitochondrial and Alzheimer's disease fields, these

results represent the first demonstration, by means of

studying two important mitochondrial markers, i.e. Bax

and cytochrome C, that mitochondrial dysfunction occurs

15 in the brains of the transgenic AD mice of the

invention. Finally, these results demonstrate that Bax

and cytochrome C are expressed in axonal structures

which are intimately associated with the A-β deposits

in the brain of the transgenic AD mouse in the same way

20 as in the brains of AD patients.

In the present invention, the mitochondrial

dysfunction is understood as being an alteration, a

modification, an overexpression or a inhibition of the

expression of the mitochondrial proteins. These

25 proteins, which preferably have a subcellular intra-

mitochondrial location, include the proapoptotic

proteins of the Bcl-2 family, such as Bax, Bak and Bad,

and the antiapoptotic proteins of the Bcl-2 family,

such as Bcl-2 and Bcl-xL, or any other mitochondrial

protein which does not belong to the Bcl-2 family and

which plays a role in apoptosis, such as citochrome C

5 and AIF, or else proteins which have recently been

recorded as being located in the mitochondria and which

can play a role in apoptosis, such as Aralar or BMCP1.

Mitochondrial proteins whose expression is

modified and which may preferably be mentioned are, in

10 particular, the Bax and/or cytochrome C proteins.

The present invention also relates to the use

of the animal model, as previously described, for

identifying compounds which can be used for treating

neurodegenerative diseases, preferably Alzheimer's

15 disease.

Thus, as a result of its advantageous

properties, which very faithfully reproduce the

characteristics of AD, this model can be used, by

contrast with the known models, to identify compounds

20 which are particularly well suited for treating AD, in

particular AD as described in man.

These compounds can be chemical molecules,

peptide or protein molecules, antibodies or chimeric

molecules, and also antisense DNAs or ribozymes.

25 The compounds which have been identified can

be used as medicaments, as such or in combination with

a pharmaceutically acceptable excipient in order to

obtain a pharmaceutical composition. The excipients

can, in particular, be sterile, isotonic saline

(monosodium phosphate, disodium phosphate, sodium

chloride, potassium chloride, calcium chloride or

5   magnesium chloride, etc., or mixtures of such salts)

solutions, or dry, in particular lyophilized,

compositions which enable injectable solutions to be

constituted by the addition, as the case may be, of

sterilized water or physiological saline. The

10   injections can be effected by the stereotactic,

topical, oral, parenteral, intranasal, intravenous,

intramuscular, subcutaneous, intraocular, transdermal,

etc. route.

        The identification of the previously

15   described compounds is based on bringing the animal

model of the invention into contact, in particular by

means of an administration, such as an injection, with

a compound or a mixture of compounds which is/are

assumed to have an action and then measuring the

20   effect(s) of the compounds, in particular at the

cerebral level of the model, on various biochemical

and/or histological changes, for example those

described in the Methods and Results sections,

including the level of production of the A-β deposits,

25   the changes linked to neurodegeneration, alteration in

the expression of mitochondrial molecules, etc.

The invention also relates to a cell which has been extracted from the previously described animal model and to its use for identifying compounds which can be used for treating neurodegenerative diseases,

5   preferably Alzheimer's disease.

The identification of previously described compounds is based on bringing cells which have been extracted from the animal model of the invention into contact with a compound or a mixture of compounds which

10   is/are assumed to have an action and then measuring the effect(s) of the compounds, at the level of the whole cells, in cell homogenates, or on a subcellular fraction, on various parameters such as cell death, production of the A-β peptide, and mitochondrial

15   activity (production of free radicals, respiratory chain, mitochondrial potential, etc.).

The results described in the examples show the advantages of the model of the invention and clearly support using this transgenic model for

20   developing therapeutic strategies such as, in particular, mitochondrial agents which resist mitochondrial dysfunction and the neuronal death which is induced by mitochondrial dysfunction.

The present invention will be described in

25   more detail with the aid of the examples, which follow and which should be regarded as being illustrative and not limiting.

LEGEND TO TABLE I: the table recapitulates the results
which were obtained in the double transgenic mouse
model exhibiting A-β deposits and markers of apoptosis
5   which is mediated by mitochondrial dysfunction in
axonal structures.

LEDEND TO THE FIGURES

Figure 1

Comparison of the levels of expression of
10 APP, of the β-secretase fragment and of A-β in various
transgenic mouse lines: 1(AA LD2(B6)), 2 (APP LD2
(FVB)), 3 (NT), 4 (Thy-1 Kozak APP_{751} SL (28)), 5 (Thy-1
Kozak APP_{751} SL (26)), 6 (Thy APP_{751} SDL (1001)), 7 (PDGF
APP_{695} SDL (46)), 8 (HMG APP SDL 20 (76)).

15

Figure 2A

Use of the antibody WO-2, which is
specifically directed against the human form of A-β,
20 for analyzing the expression of APP, of the β-secretase
12kDa fragment and of A-β in homogenates of brains of
transgenic mice which are of different ages and which
carry a single or double mutation. While the age of the
transgenic mice does not alter the level at which APP
25 is expressed, it leads to pronounced accumulation of
A-β.

Figure 2B

Analysis of the expression of human M146L PS1.

5

Figure 3

Plate illustrating the expression of human APP in the hippocampus and cortex of transgenic mice
10 carrying a single mutation (APP mutant) (b1, b2) or a double mutation (APP/PS1 mutant) (c1, c2). Note the high level of APP expression in the hippocampal (b1, c1) and cortical (b2, c2) neurons of these two lines of transgenic mice and the very low, if not
15 undetectable, level of APP expression in the corresponding cerebral regions of the nontransgenic control animals (a1, a2). In the same way, the expression of human PS1 is evident in the same cerebral regions in the transgenic mice carrying a single
20 mutation (PS1 mutant) (e1, e2) or a double mutation (APP/PS1 mutant) (f1, f2) and is undetectable in nontransgenic control animals (d1, d2).

Figures 4 and 5

25

Illustration of the A-β deposits in the brains of transgenic mice which carry a double mutation

(APP/PS1 mutant) and which are 6 months (Fig. 4) and 12 months (Fig. 5) of age. Immunohistochemical detection using several antibodies directed specifically against different epitopes of the A-β peptide: antibodies 6E10

5   for Aβ_{1-17} (Figure 4A and 5A), Dako for Aβ_{8-17} (Figure 4B and 5B), 4G8 for Aβ_{17-24} (Figure 4C and 5C), QCB for Aβ_{1-42} (Figure 4D and 5D) and FCA18 for total Aβ (Figure 4E and 5E).

10   **Figure 6**

     Pictures depicting histological sections, stained with thioflavin S (Figure 6A) and Congo red (Figure 6B)(staining green and red, respectively), at

15   the level of the hippocampal formation in transgenic mice which carry a double mutation (APP/PS1 mutant) and which are 12 months of age. Demonstration of the fibrillary conformation of the A-β deposits in the brains of these mice. Note the variable morphology of

20   the A-β deposits; spherical (arrows) or irregular (arrow heads) forms in the transgenic mice carrying a double mutation (APP/PS1 mutant).
     Figure 7

25   Progression of the A-β deposits, which were immunolabelled with the anti-Aβ antibody 4G8, in the transgenic mouse carrying a double mutation (APP/Ps1

mutant). Note that the density of the A-β deposits is
much more pronounced at 12 months of age (C1, C2, C3)
as compared with 9 and 6 months of age (B1, B2, B3 and
A1, A2, A3, respectively). Also note that the A-β

5   deposits are in the main located in a restricted
cerebral region, principally the subiculum, in the
young mouse (A1-A2), whereas they are present in the
whole of the hippocampal formation and in cortical
regions in the 12-month-old mouse (C1-C2). Figures A2,

10   B2, C2 and A3, B3, C3 depict a higher magnification of
the regions demarcated by a black frame in Figures A1,
B1, C1 and Figures A2, B2, C2, respectively. Hi:
hippocampus, Ctx: cortex.

15   **Figure 8**

     Number of A-β deposits, which were
immunolabelled with the anti-Aβ antibody 4G8, in 6 μm
thick hemibrain sections (Bregma rostrocaudal level -

20   3.4 of Franklin and Paxinos' Stereotactic Atlas) from
the double transgenic APP_{695} SDL X PS1 M1476 L mouse,
plotted against age (6, 9 and 12 months); the deposits
were quantified using an image analysis system coupled
to a colour camera and a microscope (Q600, LEICA).

Figure 9

Regional distribution of the A-β deposits in
the brain of a transgenic mouse which carries a double
5   mutation (APP/PS1 mutant) and was 12 months of age.
25 μm sections corresponding to 6 representative levels
of the rostrocaudal axis of the mouse brain were
immunolabelled with the anti-Aβ Ab 4G8. Note the very
high number of A-β deposits in the hippocampal
10 formation and all the cortical regions.

Figure 10

Quantification, in all the 12-month-old
15 transgenic mice carrying a double mutation (APP/PS1
mutant), of the A-β load, as visualized by Aβ immuno-
histochemistry (antibody 4G8) carried out on 25 μm
thick hemibrain sections (Bregma rostocaudal level
- 3.4). Note the A-β load, which reaches more than 3
20 and 1% of the total surface of the hippocampus and the
cortex (Figure 10B), respectively, and more than 9 and
5%, respectively, in the richest regions of these two
cerebral structures (Figure 10C). On the other hand,
the A-β load is less than 0.5% in the subcortical
25 regions.

1:    Dorsal cortex, comprising, in

particular, the primary visual cortex and the auditory

cortex

2:    Ventral cortex, comprising, in

5   particular, the ectorhinal cortex and the entorhinal

cortex

3:    Hippocampal formation

4:    Remainder of the hemibrain (subcortical

structures)

10

Figure 11

Figure 11A shows the presence of APP-

immunoreactive axonal structures (arrow heads in 4) in

15   the hippocampus of transgenic mice carrying a double

mutation (APP/PS1 mutant) (4) and their absence in the

hippocampus of nontransgenic control mice (1) and of

transgenic mice carrying a single APP mutation (APP

mutant) (2) or PS1 mutation (PS1 mutant) (3). In the

20   transgenic mice carrying a double mutation (APP/PS1

mutant), the APP-immunoreactive axonal structures are

also present in all the cortical regions as Figure 11B

shows, including the entorhinal cortex (a) and the

cingulate cortex (d), in the dentate gyrus and in the

25   CA1 and CA3 hippocampal regions (b, c). The regions

demarcated by a black frame in Figures b, b1 and b3 are

visualized at greater magnification in b1, in b2 and

b3, and in b4, respectively. Also note the very high
level of expression of the human APP protein in some
neuronal cell bodies (arrows in b2). The arrow in 1, 2,
3 and 4 in each case indicates the orientation of the
5   head of the dentate gyrus.

Figure 12

        Figure 12A shows the presence of PS1-
10   immunoreactive axonal structures (arrow heads in 4) in
the hippocampus of transgenic mice carrying a double
mutation (APP/PS1 mutant) and their absence in the
hippocampus of nontransgenic control mice and of
transgenic mice carrying a single APP mutation (APP
15   mutant) or PS1 mutation (PS1 mutant) (3). In transgenic
mice carrying a double mutation (APP/PS1 mutant), the
PS1-immunoreactive axonal structures are also present
in all the cortical regions as Figure 12B shows,
including the entorhinal cortex (a) and the cingulate
20   cortex (d), in the dentate gyrus and in the hippocampal
regions CA1 and CA3 (b, c, e). The regions demarcated
by a black frame in Figures c, d and e are visualized
at great magnification in c', in d' and in e',
respectively. Also note the very high level of
25   expression of the human PS1 protein in some neuronal
cell bodies in e' and d' (arrows).

Figure 13

This figure shows the presence of delta catenin-immunoreactive axonal structures in the

5   hippocampus (Hi) and the cortex (Ctx) of transgenic mice carrying a double mutation (APP/PS1 mutant) (b1, b2) and their absence in the same cerebral structures in nontransgenic control mice (a1, a2). The regions demarcated by a black frame in Figures b1 and b2 are

10 visualized at greater magnification in b1a, b1b and in b2a, b2b, respectively. Note that, just like the APP-immunoreactive and PS1-immunoreactive axonal structures, the delta catenin-immunoreactive axonal structures appear to be grouped in plaques of varying

15 size, i.e. small, medium or large.

Figure 14

Synaptophysin-immunoreactive axonal

20 structures in transgenic mice carrying a double mutation (APP/PS1 mutant) (Figure 14A) and in humans suffering from AD (Figure 14B). Note that, in doubly immunolabelled brain sections, the synaptophysin-immunoreactive axonal structures (brown

25 immunolabelling) tightly surround the A-β deposits (blue immunolabelling) in both cases.

Figure 15

SMI-immunoreactive phosphorylated
neurofilaments in transgenic mice carrying a double
5   mutation (APP/PS1 mutant) and in humans suffering from
AD. Note that, in doubly immunolabelled brain sections,
the SMI-immunoreactive phosphorylated neurofilaments
(brown immunolabelling) tightly surround the A-β
deposits (blue immunolabelling) in both cases.

10

Figure 16

tau-1 Immunoreactivity (brown
immunolabelling) concentrated in axonal structures
15 including dystrophic axons which are located around or
inside the A-β deposits (blue immunolabelling) and
neuronal cell bodies (arrow heads) in the brains of
transgenic mice carrying a double mutation (APP/PS1
mutant). Neither dystrophic axons nor cell bodies which
20 are immuno-reactive for the *tau-1* protein are present
in the non-transgenic control mice.

Figure 17

25 Activated, GFAP-immunoreactive astrocytes
(brown immunolabelling) which are present in transgenic
mice carrying a double mutation (APP/PS1 mutant)

(Figure 17A) and in humans suffering from AD (Figure

17B). Note that, in doubly immunolabelled brain

sections, the activated astrocytes surround some A-β

deposits (blue immunolabelling) in both cases.

5

Figure 18

Figures 18A to 18H show Bax expression in the

neurons of the dentate gyrus (Figure 18A, Figure 18E),

10 the CA1 (Figure 18C, Figure 18G) and CA3 hippocampal

regions (Figure 18B, Figure 18F) and the entorhinal

cortex (Figure 18D, Figure 18H)of a nontransgenic

control mouse (Figure 18A to Figure 18D) and of a

transgenic mouse carrying a double mutation (APP/PS1

15 mutant) (Figure 18E to Figure 18H). Note that, while

Bax-immunoreactive axonal structures (arrow heads) are

present in the doubly transgenic mouse, they are absent

from the nontransgenic control mouse.

Figures 18I to 18L show a double

20 immunolabelling which makes it possible to visualize

Bax expression (in brown) and the A-β deposits (in

blue) in one and the same section of the dentate gyrus

(Figures 18K and 18L) and the CA3 hippocampal region

(Figures 18I and 18J) of transgenic mice carrying a

25 double mutation (APP/PS1 mutant). Note that the Bax

immunoreactivity is concentrated in abnormal axonal

structures such as dystrophic axons which are

intimately associated with the A-β deposits (arrows).

The Bax immunoreactivity is also concentrated in some

abnormal cell bodies which appear to be strongly

immunolabelled at the level of their perinuclear

5   cytoplasm and which resemble so-called "dark" neurons

(black arrow heads). Bax immunoreactivity is also

apparent in some glial cells which are reminiscent of

activated astrocytes (white arrow heads).

10   **Figure 19**

         Bax-immunoreactive axonal structures in the

frontal cortex from a control human subject (Figure

19A) and from a patient suffering from AD (Figure 19B

15   to 19F). Note, in the AD patient but not in the control

subject, the presence of Bax-immuno-reactive axonal

structures (brown immunolabelling) such as dystrophic

axons (arrows), some of which appear to be intimately

linked to the A-β deposits (blue immunolabelling) and

20   abnormal cell bodies which exhibit very intense

labelling areas in the cytoplasm and the proximal

prolongations, thereby resembling neurofibrillary

degenerations (arrow heads).

Figure 20

Figures 20A and 20B: Pictures depicting cytochrome C expression in the CA1 hippocampal region
5   from nontransgenic control mice (Figure 20A) and transgenic mice (Figure 20B) carrying a double mutation (APP/PS1 mutant). Note the presence of cytochrome C-immunoreactive axonal structures (arrows) in the double-transgenic mouse and their absence from the
10   nontransgenic control mouse.

Figures 20C to 20H: pictures depicting a double immunolabelling which makes it possible to visualize cytochrome C expression (in brown) and the A-β deposits (in blue) on one and the same section of
15   the dentate gyrus, hippocampal regions CA1 and CA3 and the cingulate cortex from transgenic mice carrying a double mutation (APP/PS1 mutant). Note that the cytochrome C immunoreactivity is concentrated in abnormal axonal structures such as dystrophic axons
20   which are intimately associated with the A-β deposits (arrows in b, c, d, e and f). The cytochrome C immunoreactivity is also concentrated in certain abnormal cell bodies which appear to be strongly immunolabelled at the level of their perinuclear
25   cytoplasm (arrow heads in c, d and f). The high-magnification pictures in g and h demonstrate the intimate association of the cytochrome C-immunoreactive

cell structures with the A-β deposits (arrows in g and h).

Figure 21

5

Cytochrome C-immunoreactive axonal structures in the frontal cortex from a control human subject (Figure 21A) and from a patient suffering from AD (Figures 21B to 21F). Note the presence, in the AD

10 patient and not in the control subject, of cytochrome C-immunoreactive axonal structures (brown immunolabelling) such as dystrophic axons (arrows), some of which appear to be intimately linked with the A-β deposits (blue immunolabelling) and abnormal cell

15 bodies which exhibit very intense labelling areas in the cytoplasm and in the proximal prolongations, thereby resembling neurofibrillary degenerations (arrow heads).

20 **Figure 22**

Double immunofluorescent labelling making it possible to visualize Bax expression, by means of red rhodamine fluorescence (Figures 22A to C), and the

25 expression of APP, PS1 or SMI, by means of green fluorescein fluorescence (Figures 22D to F respectively), in one and the same brain section from

transgenic mice carrying a double mutation (APP/PS1

mutant). Note the presence of Bax and APP, and also of

Bax and PS1, within the same neuronal structures

(colocation in the same neurons and plaques), and, in

5 the case of control, the colocation of the Bax and SMI

immunofluorescences within or around the plaques and

not in the cell bodies. The double immunofluorescent

labelling (Figures 22G to 22H) demonstrates the

colocation of cytochrome C and delta-catenin in a

10 substantial number of neuronal structures (but not in

all of them) in the brains of the double transgenic

mice. Pictures 22I and 22J demonstrate the double

immunofluorescent labelling of Bax and APP and their

colocation in the axonal structures within or around

15 the plaques in the cerebral tissue of a human patient

suffering from AD.

Figure 23

20   Double immunofluorescent labelling making it

possible to visualize Bax expression, by means of red

CY3 fluorescence (Figures 23A to 23C), and expression

of APP, PS1 or cytochrome C, by means of green

fluorescein fluorescence (Figures 23D, 23E and 23F

25 respectively), in one and the same brain section from

transgenic mice carrying a double mutation (APP/PS1

mutant). Confirmation of the colocation of Bax and APP, of Bax and PS1 and of Bax and cytochrome C.

A/Materials and Methods

5

1. **PS1 mutagenesis**

Human PS1 was mutagenized using a Sculptor^{TM+} (Amersham, France) *in-vitro* mutagenesis system. The

10 PS1-encoding region, including a Kozak consensus motif, was subcloned into the Bluscript vector (Stratagene), and the mutations were introduced in accordance with the protocol supplied by the manufacturer by using oligonucleotides which contained the desired mutation.

15 The mutated sequences were verified by sequence analysis.

2. **Generation and identification of the PS1 M146L transgenic mice**

20

In order to construct the transgene, the cDNA encoding the mutated human PS1 was subcloned between the SmaI and BamHI restriction sites of the polylinker of the transgenic expression vector HMG (Czech et al.,

25 1997). For microinjecting, the vector sequences were removed by restricting with the enzyme NotI, and the fragment containing the expression cassette was

purified by gel electrophoresis. The purified fragment
was diluted in 10 mM Tris-HCl (pH 7.4) 0.1 mM EDTA to a
final concentration of 2.5 ng/µl and injected into one
of the two pronuclei of fertilized mouse embryos. The
5   surviving embryos were immediately transplanted into
the oviduct of adoptive (pseudopregnant) mothers. The
presence of the transgene in the neonates was
determined either by PCR or by performing a Southern
analysis. The PCR was carried out using oligomers
10 corresponding to human PS1 (5'-TAA TTG GTC CAT AAA AGG
C- 3'; 5'-GCA CAG AAA GGG AGT CAC AAG-3'), thereby
amplifying a fragment of 550 bp in size. In the case of
the Southern blot, a 1.2 kb PstI-SalI fragment from the
first intron from the HMG expression cassette, which
15 fragment was radiolabelled with alphaP-32-dCTP, was
used as a probe for detecting the transgene and the
endogenous HMG gene, as an internal control. Taken
overall, these analyses are able to exclude the
possibility of there having been any major
20 rearrangement in, or deletions of, the transgene in the
founders and their progeny.

**3.   Constructing vectors for expressing the APP
transgene**

25

3.1   PDGF APP$_{695}$SDL

The plasmid containing the PDGF promoter expression cassette was linearized, using the restriction endonuclease *Sna BI* in accordance with standard procedures (Ausubel et al.; Current Protocols in Mol. Biol.), in order to generate a blunt-ended cut for the purpose of subcloned the APP cDNA.

The mutagenesis of APP was described previously (Czech et al. 1997). The cDNA encoding human APP$_{695}$SDL was excised from the cloning plasmid using the restriction endonucleases *SmaI* and *Cla*. The cohesive ends generated by ClaI were rendered blunt-ended by treating them with the DNA polymerase Klenow fragment. The identity and integrity of the PDGF APP$_{695}$SDL construct was verified by analyzing the restriction pattern, and the junctions between the fused DNA fragments were checked by partial sequencing.

A plasmid preparation kit (Qiagen) was used for preparing the supercoiled DNA. The complete transgene was purified as described above by digesting with the restriction enzyme *NotI* and separating the transgene fragment by means of electrophoresis. The aliquots to be used for the microinjection were dialyzed against a TE buffer (10 mM Tris, pH 7.4; 0.1 mM EDTA) on a floating filter (Millipore; membrane type: VS; 0.025 μm) and then filtered (Spin-X; Costar; polyacetate membrane; 0.22 μm). The DNA was diluted to

a final concentration of 1-2 ng/μl for the

microinjection.

3.2 APP$_{751}$SDL

5

      The mutagenesis of the APP was described

previously (Czech et al. 1997), and the mutated APP

sequences were introduced into the APP$_{751}$ cDNA by

inserting the exon-8-containing Sma I/Bgl II APP

10   fragment into the Bluescript vector containing the

mutations. In order to generate the transgenic

expression construct APP$_{751}$SDL Thy-1, the APP cDNA, from

the Sma I (-95) site to the Cla I (2699) site, was

cloned into a modified pBluescript vector containing

15   SalI sites on each side of the insertion site. The

vector was digested with Sal I and the insert was

cloned into the murine Thy-1 vector using the Xho I

site (Lüthi et al. J. Neuroscience 17, 4688-4699). The

correct orientation was verified by restriction

20   analysis and the construct was sequenced at the

ligation sites. For microinjecting, the cassette was

linearized by Not I-Pvu I restriction and the fragment

containing the transgene was then purified.

25   **3.3 APP$_{751}$Kozak SL**

The mutagenesis of the APP was described previously (Czech et al. 1997), and the mutated APP sequences were introduced into the APP_{751} cDNA by inserting the exon-8-containing Sma I/Bgl II APP

5   fragment into the Bluescript vector containing the mutations. In order to optimize the translation initiation site of the APP, an optimized Kozak consensus sequence was introduced by site-directed mutagenesis carried out by means of PCR. For the PCR,

10   the oligonucleotide combination was as follows: sense oligo (initiation region): ccc ggg tcc acc atg ctg ccc ggt ttg g (Kozak sequence underlined), antisense oligo: ttc agg gta gac ttc ttg gc. The PCR product was cloned into pCR2 (Invitrogen, France), after which it was

15   sequenced and then subcloned into the $APP_{751}SL$ cDNA-containing Bluescript vector using Sma I and Acc I, thereby deleting the 5' UTR of the APP and introducing the Kozak consensus sequence. In order to generate the transgenic APP_{751} Kozak SL Thy-1 construct, the above

20   APP cDNA, extended in 3' to Cla I (2699) was subcloned into a modified Bluescript vector containing two Sal I sites on each side of the insert. The vector was digested with Sal I and the insert was cloned into the murine Thy-1 vector using the Xho I site (Lüthi et al.

25   J. Neuroscience 17, 4688-4699). The correct orientation was verified by restriction analysis, and the construct was sequenced at the ligation sites. For

microinjecting, the cassette was linearized by Not I-
Pvu I restriction and the fragment containing the
transgene was then purified.

5   **3.4   Generating transgenic animals**

The transgenic animals were obtained and
identified using already described standard procedures
(e.g. "Manipulating the Mouse Embryo"; Hogan et al. CSH
10 Press; Cold Spring Harbor, N.Y.).

4   Western blot analysis

Cerebral tissue from transgenic mice and
15 nontransgenic control mice (littermates) was
homogenized, on ice, in a 0.32 M sucrose solution
containing protease inhibitors (CompleteTM, Boehringer-
Mannheim, Germany). The cell debris was removed by
centrifuging at 1500 g for 5 min and at 4°C. The
20 protein concentration in the supernatant was measured
using the BCA protein test (Pierce, USA). For detecting
PSI, 25 µg of protein extract were incubated, at 56°C
for 20 min, in Laemmli loading buffer containing 8 mM
urea and 50 mM dithiothreitol. For detecting APP and
25 A-β, 25 µg of protein extract were denatured, at 95°C
for 10 min, in 30 µl of standard Laemmli loading
buffer. The proteins were fractionated by

polyacrylamide gel electrophoresis (SDS-PAGE). After
transferring the proteins to a nitrocellulose filter
(Amersham, France), the filter was warmed in PBS for
5 min in order to increase sensitivity and immediately
5 saturated for 1 h with 5% (w/V) skimmed milk powder in
TBST 850 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.05% (V/V)
Tween 20, and then incubated overnight at 4°C together
with the primary Ab in TBST buffer alone. The binding
of the Ab was detected using a horseradish-peroxidase-
10 conjugated anti-IgG-Ab (Amersham, France), followed by
a chemi-luminescence detection system (Amersham,
France), in accordance with the manufacturer's
instructions. The primary Ab MAB1563 (Chemicon, USA)
was used, at 1/10,000 dilution, for detecting PS1; the
15 antibody WO-2 was used, at a concentration of
0.1 µg/ml, for detecting APP and A-β.

5 **Antibodies used for the immunohistochemistry**

20 The following primary antibodies (Abs) were
used:
mouse anti-APP monoclonal Ab (1:100; 22C11,
Boehringer)
biotinylated mouse anti-Aβ_{17-24} monoclonal Ab
25 (1:200, clone 4G8, Senetek)
biotinylated mouse anti-Aβ_{1-17} monoclonal Ab
(1:200, clone 6E10, Senetek)

mouse anti-$A\beta_{8-17}$ monoclonal Ab (1:100, clone 6F/3D, Dako)

rabbit anti-$A\beta_{1-42}$ polyclonal Ab (1:300, QCB) rabbit anti-$A\beta_{total}$ polyclonal Ab (1:1000, FCA18 from

5 Checler)

rat anti-PS1 monoclonal Ab (1:50, Chemicon) rabbit anti-delta catenin polyclonal Ab (1:2000, RPR) rabbit anti-GFAP polyclonal Ab (1:3000, Dako)

rabbit anti-synaptophysin polyclonal Ab

10 (1:100, Dako)

mouse phosphorylated anti-neurofilaments (PNFs) monoclonal Ab (SMI 312, 1:100, Sternberger)

mouse anti-Tau-1 (Tau, microtubule-associated protein) monoclonal Ab (5 μg/ml, Boehringer, Mannheim)

15 rabbit anti-Bax polyclonal Ab (P19, 1:300, Santa Cruz)

mouse anti-cytochrome C monoclonal Ab (clone 7H8.2C12, 1:200, Pharmingen)

The secondary antibodies (1:400, Vector)

20 which were used were mouse anti-IgG Abs (H + L), in the case of experiments involving the use of mouse primary Abs, and rabbit anti-IgG Abs or rat anti-IgG Abs (H + L), in the case of the experiments involving the use of primary rabbit and rat Abs, respectively. In

25 some immunofluorescent labelling experiments, the secondary Ab employed was a rabbit anti-IgG Ab which was conjugated to the fluorochrome CY3 (1/400, Vector).

6   Manipulating the animals

The animals were housed under controlled temperature and humidity conditions and subjected to a 12 h day/12 h night cycle (light 7:00 EST). The animals had free access to food and water. The experiments on these animals were carried out with the agreement of the Rhône Poulenc Rorer Ethical Committee on the care and use of animals, in conformity with the standards of the "Guide for the care and use of laboratory animals" (National Research Council ILAR) and while respecting the French regulations and the EEC directive.

The animals used for the neurohistopathology studies are listed in the following table:

39

	Nontransgenic mice (littermates)					Single-transgenic mice PDGF APP$_{695}$SDL					Single-transgenic mice HMG PS1M146L					Double-transgenic mice PDGF APP$_{695}$SDL X HMGPS1M146L			
Age (months)	6	9	12	15	18	6	9	12	15	18	6	9	12	15	18	6	9	12	15
Number	1	5	4	6	2	4	2	2	4	-	1	2	9	5	7	4	5	5	-

7   Neurohistopathology

7.1   Preparing the cerebral tissue

The mice were deeply anaesthetized (Pentobarbital: 60 mg/ml/kg i.p., Ketamine: 40 mg/ml/kg i.p.) and then perfused via the heart with physiological serum and then paraformaldehyde (4% in PBS). The brains were then removed and subsequently post-fixed, at 4°C for 24 h, in the same fixing solution. After fixing, the brains were separated into right and left hemibrains, with the latter then being subjected to the standard protocol of embedding in paraffin.

The paraffin-embedded left hemibrains from the transgenic and non-transgenic mice, and also blocks of postmortem human brain tissue (frontal cortex) from a patient suffering from AD and a control subject (supplied by Dr J.P. Brion, Belgium), were sectioned, at a thickness of 6 μm (serial sections), using a microtome (LEICA RM 2155, France). The tissue blocks corresponding to the right hemibrains from the transgenic and nontransgenic mice were sectioned to a thickness of 25 μm.

For each immunohistochemical experiment, the brain sections were first of all dewaxed with xylene and dehydrated in 100% ethanol. The sections were then

incubated in H202 (1% in methanol), in order to block
endogenous peroxidase activities, rinsed in ethanol and
citrate buffer (10 Mm sodium citrate, pH 6) and finally
placed in a microwave oven (650W, Whirlpool) for
5   2 × 5 min in the citrate solution. For the experiments
in which the A-β protein was immunolabelled, the
sections were subjected to an additional step, i.e. a
3 min incubation in 80% formic acid.

10   **7.2   Thioflavin S**

The sections were stained with 1%
thioflavin S (Sigma, France) after having been
incubated for 10 min in Mayer's haematoxylin solution
(Sigma, France) in order to block nuclear fluorescence.
15   An FITC filter was then used to observe them in a
microscope equipped with a fluorescence system
(Axioscop Zeiss, France).

7.3. Congo Red

20   A polarized light system was used to analyze
the sections which were stained with Congo red (Amyloid
staining kit, Accustain, Sigma, France). Only the
birefringent (to the rotation of the polarizer) red
deposits were regarded as being Congo red-positive
25   deposits.

7.4. Immunoenzymic labelling

After having been incubated for 30 min in blocking buffer (10% normal goat serum (Chemicon) in PBS containing 0.1% triton (Sigma)), the dewaxed brain sections were incubated in the solution of the primary

5 Ab (overnight at 4°C). After having been rinsed several times, the sections were brought into contact with the biotinylated secondary Ab (at ambient temperature for 2 h) and then into contact with the avidin-biotin peroxidase complex in accordance with the

10 manufacturer's (Vectastin ABC kit, Vector Laboratories, Burlingame, CA) instructions. 3-3'-Diaminobenzidine was used as the chromogen for the peroxidase enzyme. For the preabsorption experiments, the anti-Bax Ab (antibody P19, Santa Cruz) was incubated with the

15 synthetic Bax peptide (Control peptide P19, Santa Cruz) (tested concentrations of the peptide: 0.002, 0.02 and 0.2 mg/ml) for at least 12 h before being used in accordance with the previously described immunohistochemical protocol. The anti-cytochrome C Ab

20 (7H8.2C12, Pharmingen) was incubated, using the same protocol, with exogenous purified cytochrome C from horse or rat heart (Sigma) (tested concentrations of the purified proteins: 0.01 and 0.1 mg/ml).

25 **7.5. Quantifying the amyloid load**

The deposition of A-β in Aβ-immunolabelled (biotinylated mouse anti-Aβ$_{17-24}$, monoclonal Ab 4G8,

Senetek) 25 μm hemibrain sections was quantified using diaminobenzidine as the chromogen and an image analysis system coupled to a color camera and a microscope (Q600 system, LEICA). Video images of each anatomical region

5 of interest were captured and a threshold was defined for automatically detecting the mean gray level corresponding to the immunolabelling of the A-β deposits (and capable of distinguishing the specific labelling from the background noise). The experimenter

10 checked each field manually in order to eliminate any artifact by manual means. In each mouse, the A-β load was measured at the bregma rostrocaudal level -3.4 of the mouse stereotactic atlas (Franklin and Paxinos). The load of A-β is defined as being the percentage of

15 the area of A-β immunolabelling with respect to the total area of the cerebral region analyzed, that is the hippocampus, the cortex and the remainder of the section (subcortical regions).

20 **7.6. Double immunoenzymic labelling**

The immunohistochemical double-labelling experiments were carried out by incubating the brain sections in accordance with a two-step protocol. In brief, the sections were immunolabelled in a first step

25 using a primary Ab (for example: anti-GFAP, anti-synaptophysin, anti-Bax or anti-cytochrome C), with this antibody being visualized by means of a brown

labelling obtained using diaminobenzidine (enzymic

substrate for the horseradish peroxidase) in accordance

with the previously described protocol. The same

sections were then immunolabelled using the primary

5   anti-Aβ_{17-24} Ab (mouse monoclonal Ab 4G8, Senetek), with

this antibody being visualized by means of a blue

labelling obtained using the horseradish peroxidase

substrate SG (SG peroxidase substrate kit, Vector).

10   **7.7. Double immunofluorescent labelling**

A double immunofluorescent labelling was

performed in some experiments, in particular with the

aim of showing whether Bax colocates with APP, PS1,

cytochrome C or SMI in axonal structures associated

15   with the A-β deposits in transgenic mice carrying a

double mutation (APP/PS1 mutant). In brief, the

sections were first of all incubated in the anti-Bax Ab

and then visualized either using a secondary

biotinylated rabbit anti-IgG Ab which was conjugated to

20   CY3 (1:400, Chemicon) or using a secondary rabbit

anti-IgG Ab and then a signal amplification kit

(Streptavidin-peroxidase/tetramethyl-Rhodamide Tyramide

complex) in accordance with the manufacturer's (New

England Nuclear) instructions. For the second

25   immunofluorescent labelling, the sections were

incubated consecutively with a primary anti-APP,

anti-PS1, anti-cytochrome C or anti-SMI Ab and then

with a secondary biotinylated mouse or rat anti-IgG Ab.
The sections were finally visualized using a second
signal amplification system (Streptavidin-peroxidase/
Fluorescein Tyramide complex) in accordance with the

5   manufacturer's (New England Nuclear) instructions. The
sections were observed under the microscope using CY3
or rhodamine filters (excitation at 550 nm, emission at
570 nm) in the case of the Bax immunofluorescent
labelling and a fluorescein filter (excitation at

10   495 nm, emission at 517 nm) in the case of the second
immunofluorescent labelling. In the same way, in the
case of the double immunofluorescent labelling
experiments which were performed with the aim of
showing whether cytochrome C colocates with the delta-

15   catenin protein, the sections were firstly incubated in
the anti-cytochrome C Ab and then visualized using the
Rhodamine fluorescence system. In a second step, these
same sections were incubated in the anti-delta catenin
Ab and visualized using the Fluorescein fluorescence

20   system as described above.

EXAMPLES

EXAMPLE 1: Analysis of the levels at which the

25   transgene is expressed

1.1   Comparison of the levels at which the transgene is expressed in different transgenic mice expressing mutated APP

Brain homogenates from different transgenic
5   mouse lines (different expression constructs) were analyzed for APP expression by means of Western blotting using a WO-2 monoclonal antibody. This antibody is specially indicated since it recognizes human APP and A-β but does not recognize the endogenous
10   mouse APP. Thus, no signal was detected in the lane corresponding to the nontransgenic mouse (Fig. 1, lane 3). Lanes 1 and 2 in Figure 1 correspond to brain extracts from transgenic mice which were previously shown to express high levels of the APP transgene
15   (Moechard et al. 1999a). The transgenic PDGF $APP_{695}SDL$ mice in lane 7 were used to produce double transgenic mouse lines together with the mice carrying the PS1 M146L transgene. A moderate level of APP expression can be seen in the mice having the HMG promoter in the
20   construct (Czech et al. 1997). Note that, in the mice which are transgenic for the Swedish mutation form of APP, the beta-secretase fragment (12 KDa) is increased relative to the complete APP holoprotein.

25   **1.2   Western blot analysis of the expression, and the processing, of the transgene in the brains of APP and Presenilin double transgenic mice**

In order to find out whether the expression

of each individual transgene was modified in the mice

carrying the two transgenes (obtained by crossing),

homogenates of brains from double transgenic mice of

5   different ages were analyzed for expression of APP and

A-β by means of Western blotting using the WO-2

monoclonal antibody (described above). No difference in

the intensity of the bands corresponding to the

complete 100 kDa APP sequence and the 12 kDa

10 aminoterminal fragment is visible between the

PDGF-APP$_{695}$SDL (monotransgenic) mice and the

APP$_{695}$SDL × PS1M146L double-transgenic mice (Fig. 2A,

compare also with lane 7, Fig. 1). In addition, the

ages of the transgenic mice do not have any effect on

15 the expression of the APP transgene or on its

metabolism at the level of the beta cleavage site.

However, A-β accumulates strongly at 9 months, with

this accumulation beginning at the age of 6 months.

This increase in A-β correlates well with the beginning

20 of the formation of amyloid plaques in the brains of

the transgenic mice (see below). This suggests that the

increase in A-β which is detected by Western blotting

may correspond to the A-β which has accumulated in the

amyloid plaques.

25 Another Western blot analysis was performed on brain

homogenates in order to determine whether the human PS1

protein is expressed in the double-transgenic mice and

in the nontransgenic (littermate) control mice. A
monoclonal Ab was used which was directed against the
aminoterminal part of PS1. This Ab is specific for
human PS1; no signal for the endogenous PS1 protein is

5   detected in the cerebral homogenate from the single-
mutant APP mouse (Fig. 2B, lanes 1 and 2). The
characteristic aminoterminal PS1 fragment is detected
in the brain of the mouse expressing PS1M146L (Fig_2B,
lanes 3-7), with the appearance of the complete

10 PS1M146L holoprotein (approx. 51 kDa), probably due to
saturation of the presenilin processing, as has
previously been described in transgenic rats and mice
expressing high quantities of PS1 (Czech et al., 1998;
Thinakaran et al., 1996). The expression and processing

15 of human PS1 in the double-transgenic mouse do not vary
with the age of the animal.
In conclusion, the levels at which the individual
transgenes (APP or PS1) are expressed are simular in
the monotransgenic and double-transgenic mice.

20

**EXAMPLE 2 Location and regional expression of the
transgenes in single (PDGF APP$_{695}$SDL or HMG PS1M146L) or
double (PDGF APP$_{695}$SDL x HMG PS1M146L) mice.**

The immunohistochemical experiments on APP,

25 using the mouse monoclonal Ab (Ab 22C11) directed
against the APP protein, demonstrated that single-
transgenic (PDGF APP$_{695}$SDL) and double-transgenic

(PDGF APP$_{695}$SDL × HMG PS1M146L) mice express the APP

protein more strongly than do nontransgenic control

mice (Fig. 3). This result confirms that these two

transgenic mouse lines express the human APP protein

5   encoded by the APP$_{695}$SDL transgene. The human APP is

located exclusively in the neurons. Its pattern of

expression is similar in the two transgenic mouse lines

and involves a large number of cerebral structures,

with the highest level of expression being observed in

10   the neuronal layers of the hippocampal formation

(Fig. 3), followed by a similar level of expression in

the cortical regions, such as the entorhinal cortex,

and in the amygdala. The subcortical regions exhibit a

lower level of expression of the transgene, and the

15   human APP protein is not detected either in the glial

cells or at the vascular level.

     In the same way, the immunohistochemical

experiments on PS1, using the rat monoclonal Ab

directed against the human PS1 protein (Ab which

20   specifically recognizes human PS1), demonstrated that

the single-transgenic (HMG PS1M146L) and double-

transgenic (PDGF APP$_{695}$SDL × HMG PS1M146L) mice express

the human PS1 protein in the brain with specific

location in the neurons (Fig. 3), although some

25   immunolabelled glial cells are occasionally observed.

The pattern of expression of the human PS1 protein is

similar in the two transgenic mouse lines. Its level of

expression is elevated in the cortical structures such
as the hippocampal formation (Fig. 3) and different
regions of the cortex, and also in the subcortical
regions. The protein is not expressed in the white

5   substance. Both in the case of the human APP protein
and in the case of the human PS1 protein, neither the
pattern nor the level of expression changes with age
(from 6 to 12 months).

10 **EXAMPLE 3 Demonstration that the process of amyloid
deposition is accelerated in the brains of double-
transgenic mice (PDGF APP$_{695}$SDL × HMG PS1M146L) which
are carrying both the mutant APP protein and the mutant
PS1 protein.**

15

We used both a variety of anti-Aβ Abs (see
Materials and Methods) and conventional histological
markers (Thioflavin S and Congo Red), which were known
to stained A-β deposits in human tissues from patients

20 suffering from AD, for showing whether the different
transgenic mouse models which had been generated
developed A-β deposits in the brain.

Using the set of A-β deposit markers, we
showed that none of the single-transgenic mice, i.e.

25 PDGF APP$_{695}$SDL or HMG PS1M146L, exhibited any amyloid
deposition in the brain at the ages of 3, 6, 9, 12 and
15 months. On the other hand, the double-transgenic

PDGF $APP_{695}SDL$ × HMG PS1M146L mice exhibited A-β

deposits from the age of 6 months onwards. The various

anti-Aβ Abs employed all demonstrate the existence of

this process of amyloid protein deposition (Figs. 4 and

5   5). These A-β deposits are also stained with

Thioflavin S and Congo Red, thereby confirming their

fibrillary conformation (Fig. 6).

EXAMPLE 4: Progression and regional pattern of amyloid

10   **deposition in the double-transgenic (PDGF $APP_{695}SDL$ ×**

HMG PS1M146L) mice

With the aim of assessing the progression and

pattern of regional distribution of the amyloid

deposition resulting from transgene expression in the

15   double-transgenic mice (PDGF $APP_{695}SDL$ × HMG PS1M146L),

the number of A-β deposits in 6 μm-thick hemibrain

sections at different ages, and the amyloid load in

25 μm-thick sections at 12 months of age, were

quantified using the anti-Aβ Ab 4G8. We focused on the

20   cortex and hippocampus for quantifying the amyloid

load, since these two cerebral structures are involved

in an early and predominant manner in the

neuropathology of the transgenic mice and patients

suffering from AD.

25   The number of A-β deposits is higher in the

12-month-old mice than in the mice aged 6 and 9 months,

as Figures 7 and 8 demonstrate qualitatively and

quantitatively, respectively. At 6 and 9 months, the

A-β deposits are located in a limited cerebral region

corresponding to the subiculum and to the dorsal part

of the hippocampal region CA1 (Fig. 7). At 12 months,

5   on the other hand, the more numerous A-β deposits are

located over the whole of the hippocampal and cortical

regions along the whole length of the rostrocaudal axis

of the brain (Fig. 7 and Fig. 9). In 12-month-old mice,

the A-β deposits are only occasionally present in some

10   subcortical structures (for example the internal

capsule, the dorsolateral thalamus and the basal

ganglia). They are not present in either the cerebellum

or in the spinal cord. Quantitative analysis of the

amyloid load in 12-month-old mice (Fig. 10)

15   demonstrates that the percentages of the Aβ

immunolabelling area with respect to the total area of

the analyzed region reach values of 3.1; of 0.8 to 1.1,

and of < 0.5 for the hippocampus, the cortex and the

remainder of the hemibrain, respectively. The amyloid

20   load reaches more than 9% and 5% in the richest areas

of the hippocampus and cortex, respectively. These

amyloid loads are of a similar order of magnitude to

those previously described in the human AD brain

(6-12%) (Hyman et al., 1993).

25

EXAMPLE 5: Axonal structures in the double-transgenic

(PDGF APP$_{695}$SDL × HMG PS1M146L) mouse

In all the transgenic mice which develop A-β
deposits, the deposits of large and medium size contain
numerous axonal structures, as is demonstrated, on the
5   one hand, by the pictures of single immunolabellings
using different markers for dystrophic axons, such as
APP (Fig. 11), PS1 (Fig. 12) and delta-catenin
(Fig. 13), and, on the other hand, by the pictures of
double immunolabellings, which visualize the A-β
10   depositions, using the anti-Aβ Ab 4G8, and the axonal
structures, using the anti-synaptophysin Ab or the
anti-SMI Ab, in one and the same brain section (Fig. 14
and Fig. 15, respectively). As Figures 14 and 15 show,
synaptophysin-immunoreactive and SMI-immunoreactive
15   axonal structures are present in the brains of the
double-transgenic mice and are intimately associated
with the A-β deposits. Furthermore, our results
demonstrate that the axonal structures are very similar
to those described in the human AD brain.
20   In order to show whether neurofibrillary
degenerations are also present in the brains of the
double-transgenic mice, a double immunolabelling was
performed for the purpose of visualizing the A-β
deposits, using the anti-Aβ Ab 4G8, and the
25   neurofibrillary degenerations, using the anti-*Tau-I* Ab,
in one and the same brain section. As Figure 16 shows,
the anti-*Tau-I* Ab labels axonal structures such as the

axons which carpet the A-β deposits and the abnormal
cell bodies. While these two types of immunolabelled
structure are present in the cerebral regions which are
rich in A-β deposits (hippocampal formation and

5   cortex), they are absent from the subcortical regions
which are poor in, or lack, A-β deposits.

Taken as a whole, these results clearly
demonstrate that, in addition to the phenomenon of
amyloid deposition, degenerative processes take place

10   within the cognitive structures of the double-
transgenic mice in a manner similar to those in the AD
brain.

EXAMPLE 6: Presence of activated astrocytes within or

15   **around the amyloid deposits in the double-transgenic**

(PDGF APP$_{695}$SDL × HMG PS1M146L) mouse

Inasmuch as the A-β deposits in the AD
cerebral tissue are associated with glial cells (the

20   inflammatory cells of the central nervous system), an
investigation was carried out to determine whether a
glial reaction takes place in the neuropil of our
transgenic mice which develop A-β deposits. No
GFAP-immunoreactive reactive astrocyte was observed in

25   the brains either of the nontransgenic control mice or
of the transgenic mice which did not develop A-β
deposits (for example the single-transgenic

PDGF APP$_{695}$SDL or HMG PS1M146L mice). On the other hand, all the double-transgenic mice, having A-β deposits in their brains, possess GFAP-immunoreactive reactive astrocytes which are intimately associated with the A-β
5   deposits (Fig. 17).

EXAMPLE 7: Demonstration of mitochondrial dysfunction in the double-transgenic (PDGF APP$_{695}$SDL × HMG PS1M146L) mouse

10

A single immunolabelling was carried out in order to visualize the Bax protein on its own, and a double immunolabelling was carried out in order to visualize the Bax protein and the A-β deposits in one
15 and the same brain section; this was done with the aim of establishing a relationship between Bax expression and the amyloid plaques.

The results of the single labelling showed that the Bax protein is expressed in the neurons of a
20 large number of cerebral structures, such as the neuronal layers of the hippocampal formation and all the cortical regions (Fig. 18A to H), both in the non-transgenic control mice and in the transgenic mice. However, only the double-transgenic
25 (PDGF APP$_{695}$SDL × HMG PS1M146L) mice, and not the single-transgenic (PDGF APP$_{695}$SDL × HMG PS1M146L) mice, or the nontransgenic control mice, exhibit Bax

immunoreactivity in cellular structures such as

dystrophic axons (Fig. 18A to H), neuronal cell bodies

resembling early neurofibrillary degenerations, and,

occasionally, activated glial cells (see below).

5 Interestingly, these abnormal Bax-immunoreactive cell

structures are located exclusively in cerebral regions

which are rich in A-β deposits, such as the dentate

gyrus, hippocampal regions CA1 and CA3, the subiculum

and the cingulate and entorhinal cortices.

10   The double immunolabelling with the anti-Bax

and anti-Aβ Abs subsequently confirmed that the Bax

immunoreactivity in the axonal structures and the

activated glial cells is intimately associated with the

A-β deposits in the double-transgenic animals

15 (Figs. 18I to L). These Bax-immunoreactive cellular

structures were never detected in the brains of the

non-transgenic or single-transgenic animals. The Bax

immunolabelling in these studies is specific, as the

preabsorption experiments using a blocking peptide

20 demonstrated.

The results from the single immunolabelling

of cytochrome C showed that the cytochrome C protein is

expressed in the neuronal cell bodies in the 4 groups

of animals examined (nontransgenic controls, and

25 single- and double-transgenic animals - Table I). The

pattern of the distribution of the cytochrome C-

immunoreactive neurons is similar to that described for

Bax. Interestingly, cytochrome C is shown, as in the

case of Bax, to be expressed in cell structures such as

axons and abnormal neuronal cell bodies (Fig. 20) only

in the double-transgenic mouse. These cytochrome C-

5   immunoreactive cell structures are never observed in

the single-transgenic or nontransgenic control mice

(Fig. 20). In the double-transgenic mice, the

cytochrome C-immunoreactive axonal structures, like the

Bax-immunoreactive axonal structures, are located in

10   the cerebral structures where the A-β deposits are

found, i.e. chiefly the dentate gyrus, hippocampal

regions CA1 and CA3 and the cingulate cortices

(Fig. 20). The double immunolabelling of the

cytochrome C and the A-β deposits shows that, in these

15   double-transgenic animals, the cytochrome C-

immunoreactive axonal structures are intimately

associated with the amyloid plaques (Fig. 20). The

cytochrome C immunolabelling is specific, just like the

Bax immunolabelling, as the preabsorption experiments

20   using purified cytochrome C proteins demonstrated.

In parallel with the experiments performed on

the transgenic mice, the Bax/Aβ and cytochrome C/Aβ

double immunolabellings were carried out on postmortem

human brain tissue from a control individual and from

25   an AD patient. The results obtained (Fig. 19 and

Fig. 21) show that, in the human AD brain, the

Bax-immunoreactive or cytochrome C-immunoreactive

axonal structures such as dystrophic axons and early

neurofibrillary neurodegenerations are present within

or around the amyloid plaques. The Bax-immunoreactive

and cytochrome C-immunoreactive abnormal neuronal

5   structures were not observed in the control human

tissue.

In order to confirm the presence of the Bax

and cytochrome C proteins in abnormal axonal structures

in the brains of double-transgenic mice, double

10   immunofluorescent labellings were also performed which

demonstrated that Bax was colocated with APP, PS1 and

cytochrome C in the same axonal structures which carpet

the A-β deposits (Figs. 22 and 23). Finally, it was

shown that cytochrome C is colocated with delta-catenin

15   in these same axonal structures (Fig. 22).

The fact that Bax and cytochrome C are

overexpressed in axonal structures such as axons and

abnormal neuronal cell bodies both in the human AD

brain and in the brains of the double-transgenic mice

20   demonstrates the involvement of these proteins in

regulating early events associated with neuronal death

in the AD patient and in this transgenic mouse model.

These findings in the mouse are the first to

show that the expression of two different mitochondrial

25   proteins, i.e. Bax and cytochrome C, which are known to

induce cell death in culture (see introduction), is

altered in the brains of transgenic mice which develop

A-β deposits. This modified expression of Bax and

cytochrome C consists in these two mitochondrial

markers accumulating in abnormal neuronal structures

which are intimately associated with the amyloid

5   plaques.

An important point is that the

Bax-immunoreactive and cytochrome C-immunoreactive

axonal structures in the double-transgenic mouse

resemble those observed in the human AD brain,

10 indicating that the same type of mitochondrial

dysfunction is found in the human AD patient.

Aβ deposition and markers of apoptosis mediated by mitochondrial dysfunction in axonal structures in the double-transgenic (PDGF APP$_{695}$SDL X HMG PS1M146L) mouse

Table I

	Nontransgenic mice (littermates)					Single-transgenic mice PDGF APP$_{695}$SDL					Single-transgenic mice HMG PS1MI146L					Double-transgenic mice PDGF APP$_{695}$SDL X HMGPS1M146L			
Age (months)	6	9	12	15	18	6	9	12	15	18	6	9	12	15	18	6	9	12	15
Expression of the transgene (APP/PS1)	-/-	-/-	-/-	-/-	-/-	+/-	+/-	+/-	+/-		-/+	-/+	-/+	-/+	-/+	+/+	+/+	+/+	
A-β deposits	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+++	
Bax	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+++	
Cytochrome C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+++	

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CLAIMS

1.   Non-human transgenic animal model of Alzheimer's disease which exhibits both amyloid plaques and mitochondrial dysfunction.

2.   Animal model according to Claim 1, characterized in that it coexpresses the β-amyloid peptide precursor (APP) and a presenilin, preferably presenilin 1 (PS1).

3.   Animal model according to Claim 2, characterized in that it coexpresses mutated forms of APP and/or PS1.

4.   Animal model according to Claim 3, characterized in that the mutation in the APP gene is selected from the "Swedisch", "London" and "Dutch" mutations, taken on their own or in combination.

5.   Animal model according to Claim 3, characterized in that the mutation in the PS1 gene is selected from the M146L, A246E, C410Y, H163R, L286V and L235P mutations, taken on their own or in combination.

6.   Animal model according to Claim 5, characterized in that it is the M146L mutation.

7.   Animal model according to Claim 1, characterized in that the mitochondrial dysfunction is an alteration, a modification, an overexpression or an inhibition of the expression of the mitochondrial proteins.

8.   Animal model according to Claim 7,
characterized in that the proteins are
intramitochondrial proteins.

9.   Model according to Claim 8,
5   characterized in that the proteins are the BAX and/or
cytochrome C proteins.

10.   Use of the animal model as described in
accordance with Claims 1 to 9 for identifying compounds
which can be used for treating neurodegenerative
10 diseases, preferably Alzheimer's disease.

11.   Cell which is extracted from an animal
model as described in accordance with Claims 1 to 9.

12.   Use of a cell as described in accordance
with Claim 11 for identifying compounds which can be
15 used for treating neurodegenerative diseases,
preferably Alzheimer's disease.

As printed

(54) Title: NOVEL ANIMAL MODEL OF ALZHEIMER DISEASE WITH AMYLOID PLAQUES AND MITOCHONDRIAL DYSFUNCTIONS

(54) Titre: NOUVEAU MODELE ANIMAL DE LA MALADIE D'ALZHEIMER PRESENTANT A LA FOIS DES PLAQUES AMYLOIDES ET DES DYSFONCTIONNEMENTS MITOCHONDRIAUX

(57) Abstract: The invention concerns the field of transgenic animal models and more particularly, animal models of Alzheimer disease. The invention relates to a novel animal model of Alzheimer disease having both amyloid plaques and mitochondrial dysfunctions.

(57) Abrégé: La présente invention concerne le domaine des modèles animaux transgéniques et plus particulièrement, les modèles animaux de la maladie d'Alzheimer. L'invention se rapporte à un nouveau modèle animal de la maladie d'Alzheimer présentant à la fois des plaques amyloïdes et des dysfonctionnements mitochondriaux.

WO 01/20977 A1
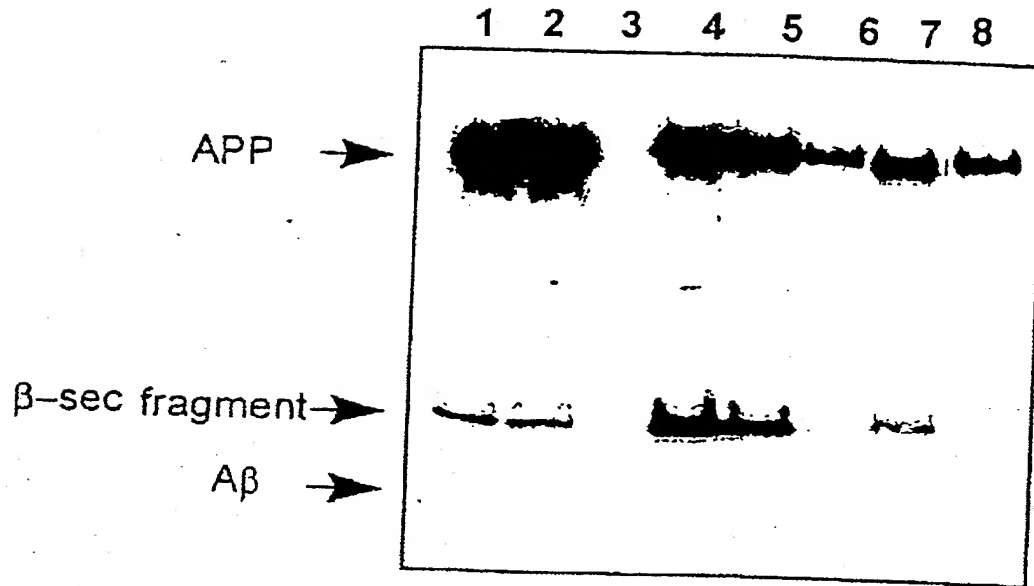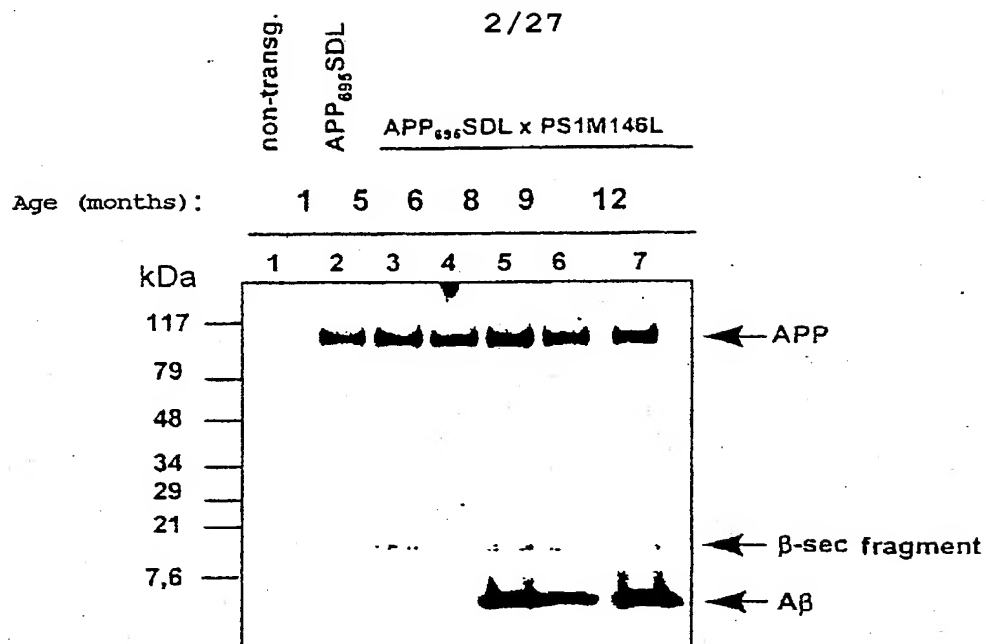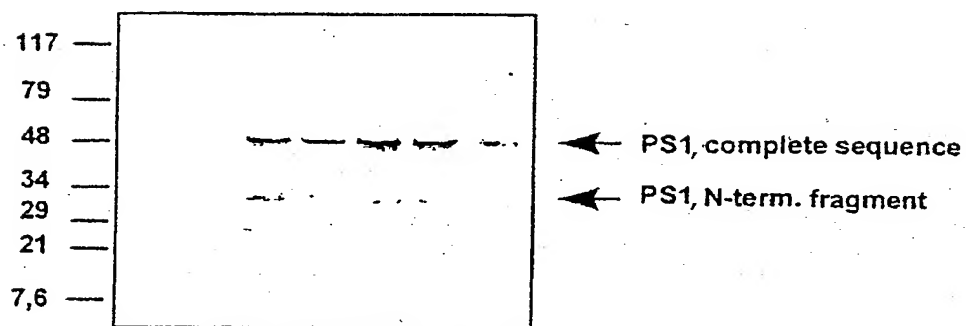
Figure 1

Figure 2A

Figure 2B

c2

f2

c1

f1

b2

e2

b1

e1

a2

d2

a1

d1
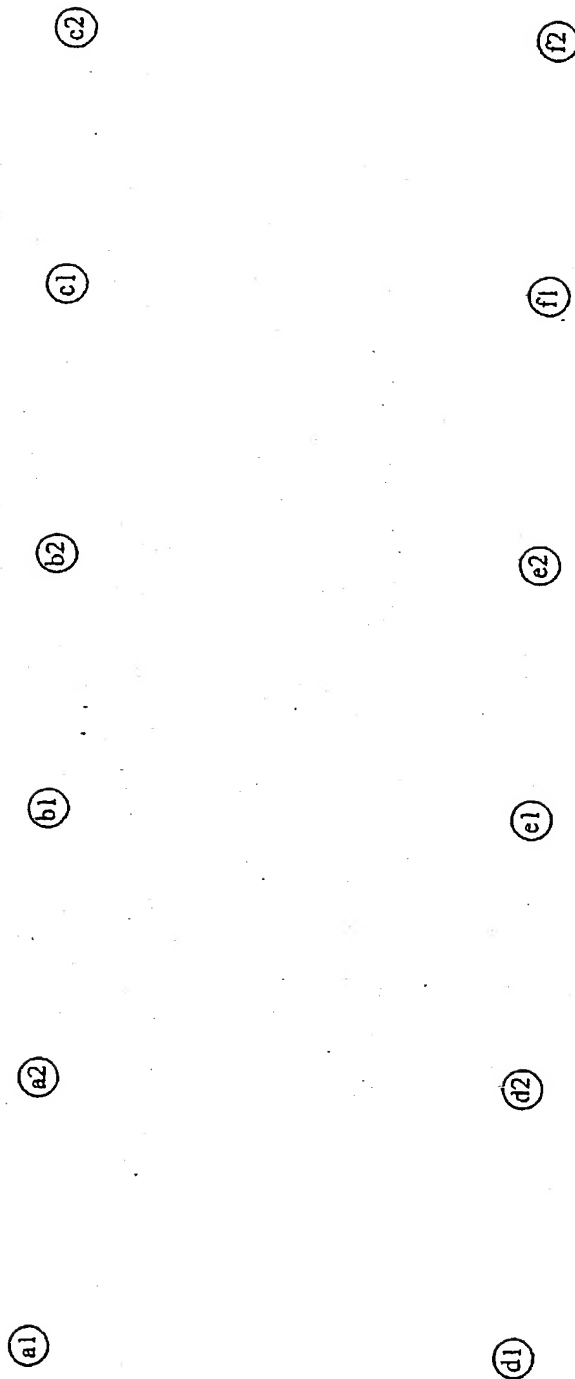
Figure 3

Figure 4C

Figure 4E

Figure 4B

Figure 4A

Figure 4D

Figure 5C

Figure 5E

Figure 5B

Figure 5A
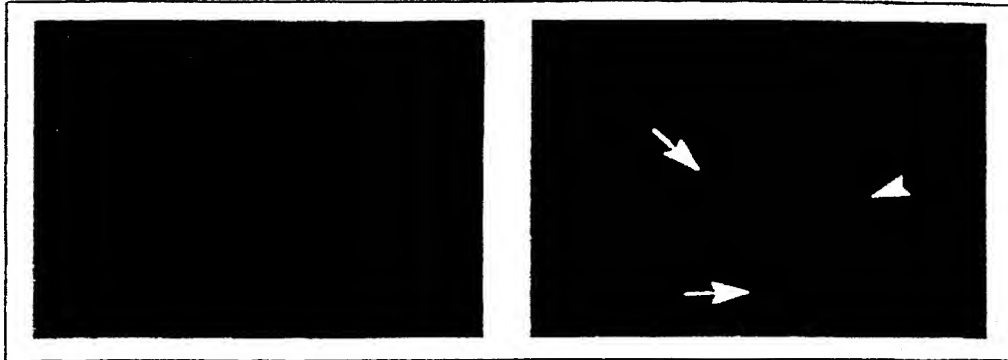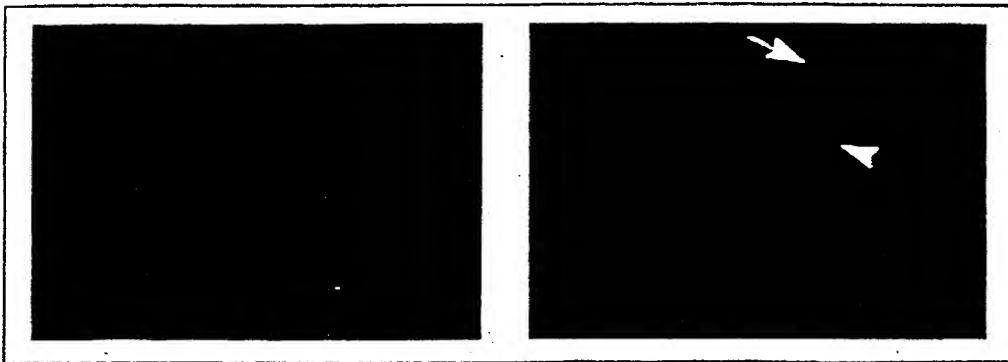
Figure 5D

Figure 6A

Figure 6B

Figure 7

Figure 8

Rostrocaudal levels vs. Bregma
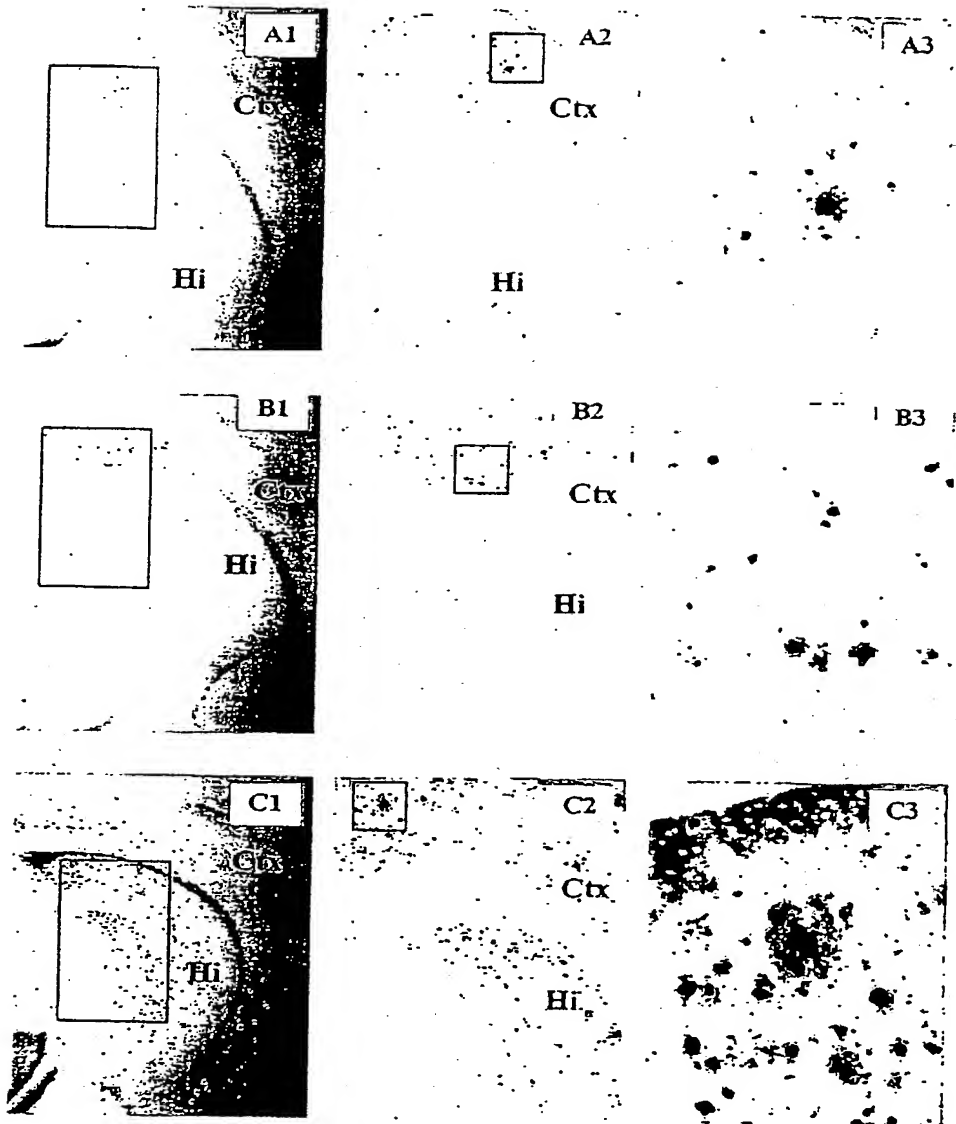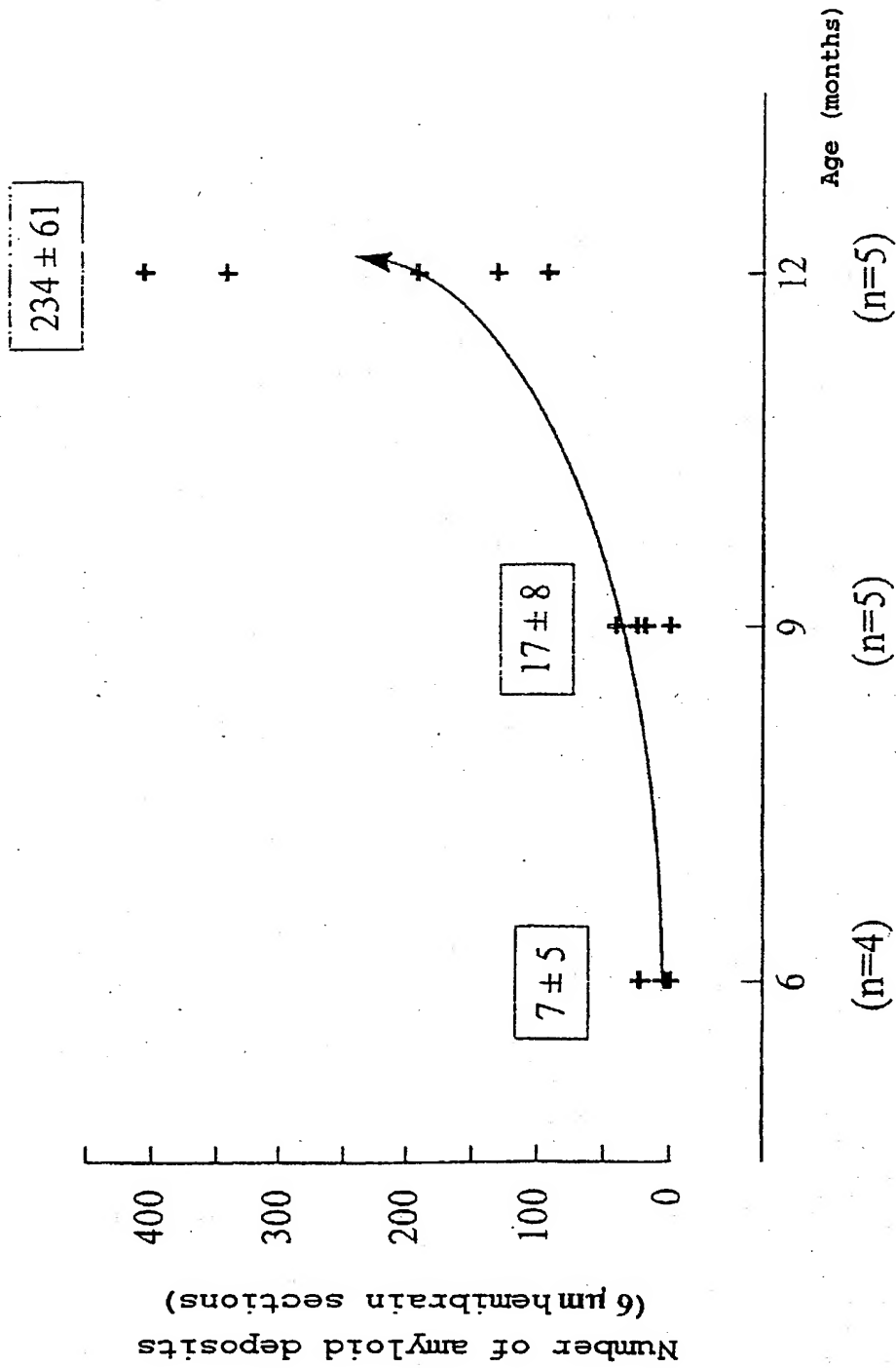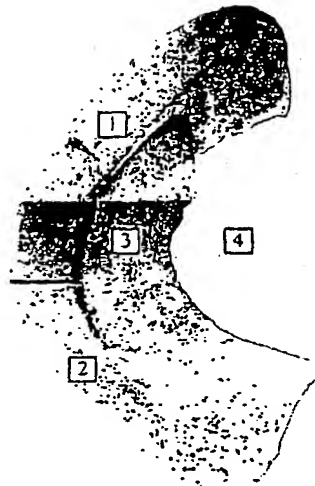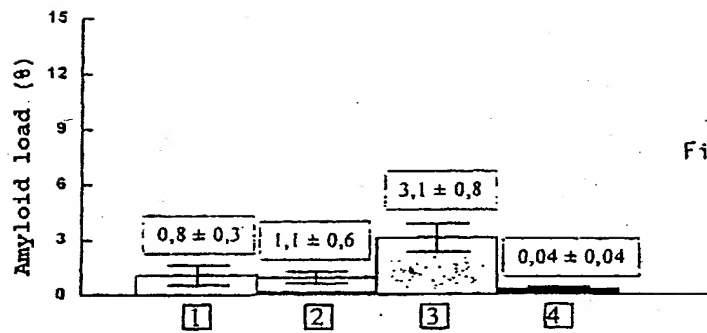
Figure 9

Figure 10A

Figure 10B

Figure 10C

Figure 10
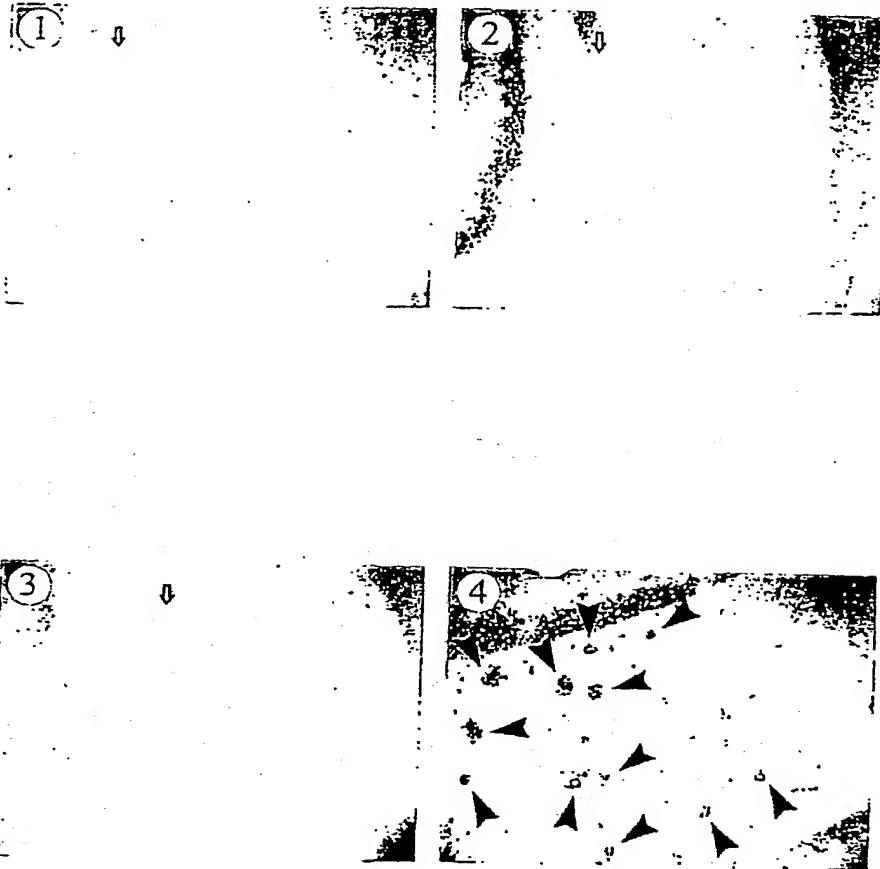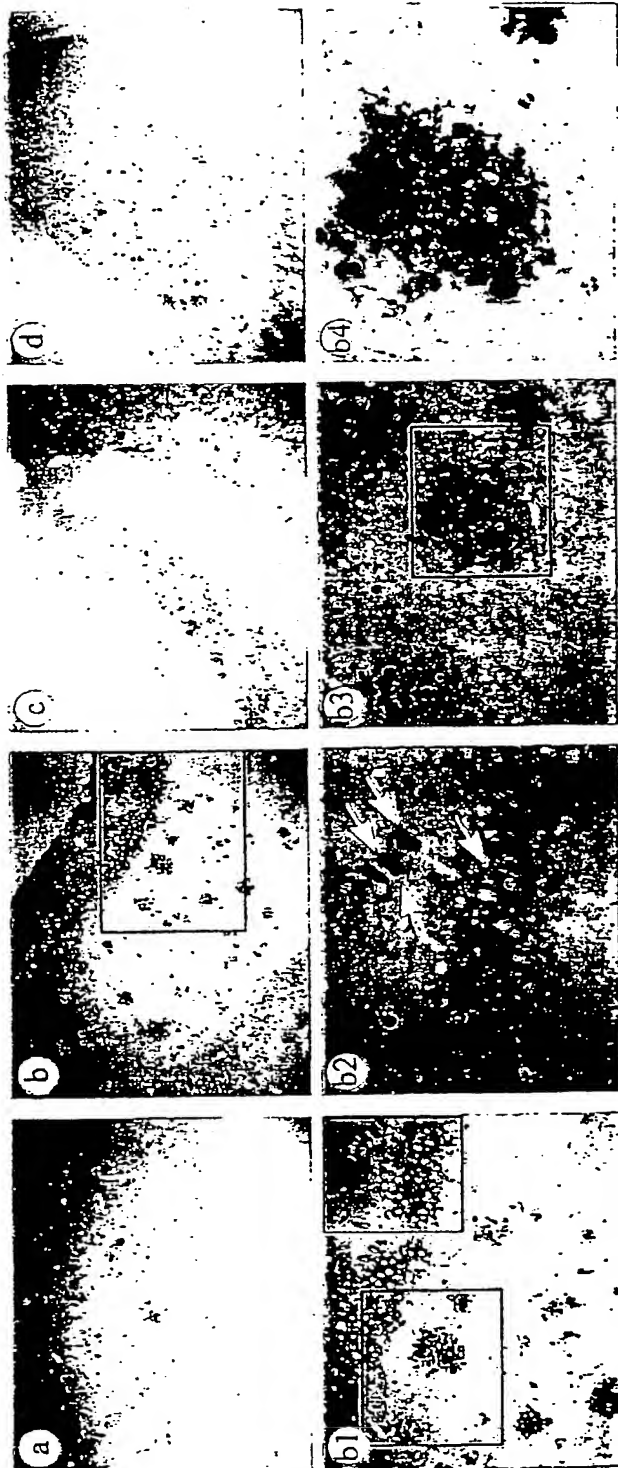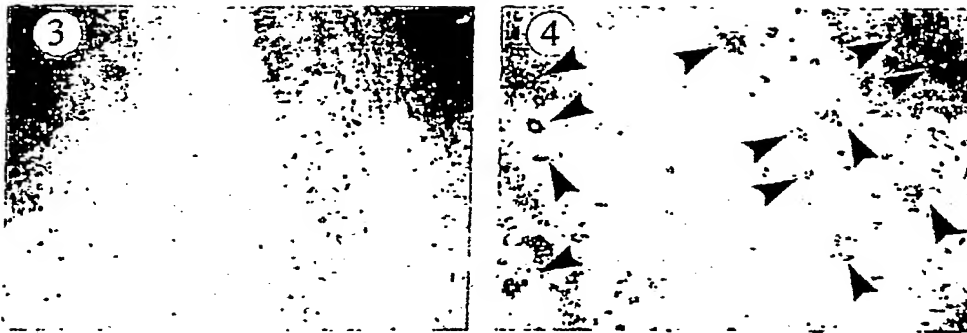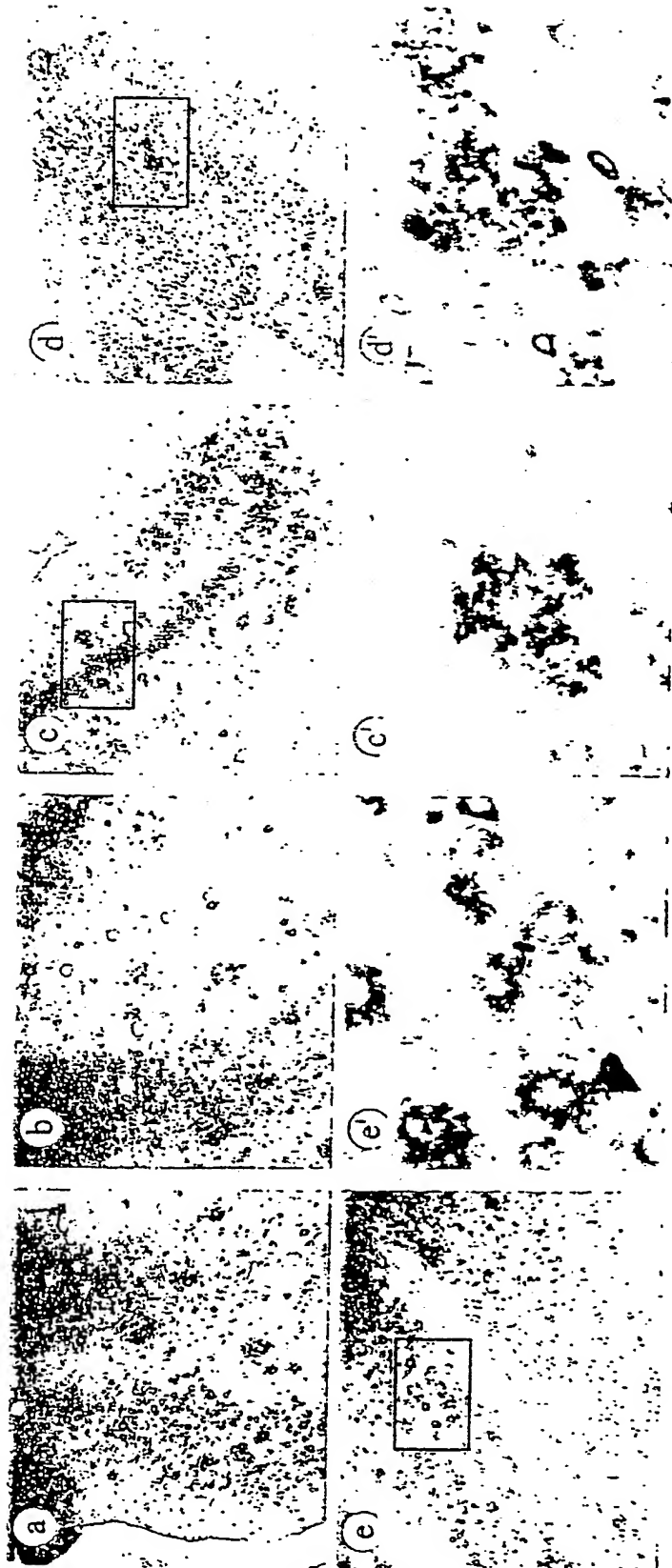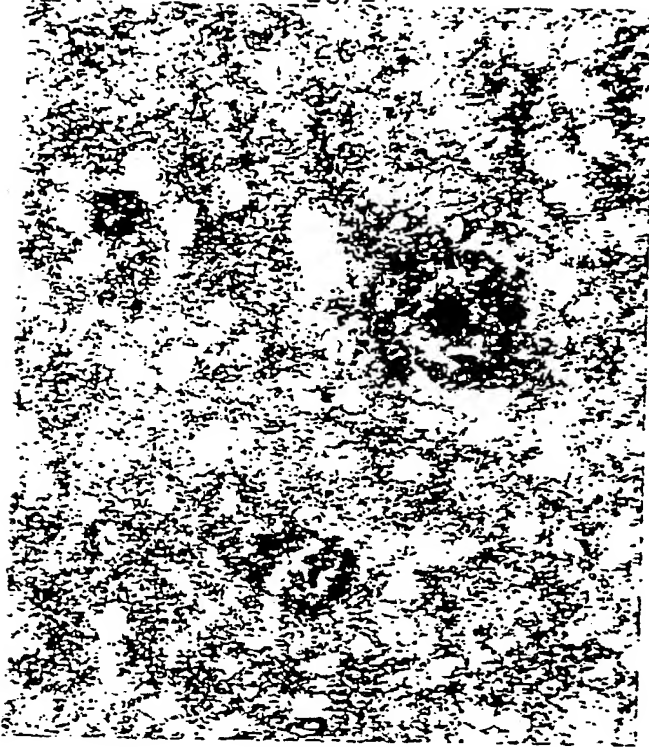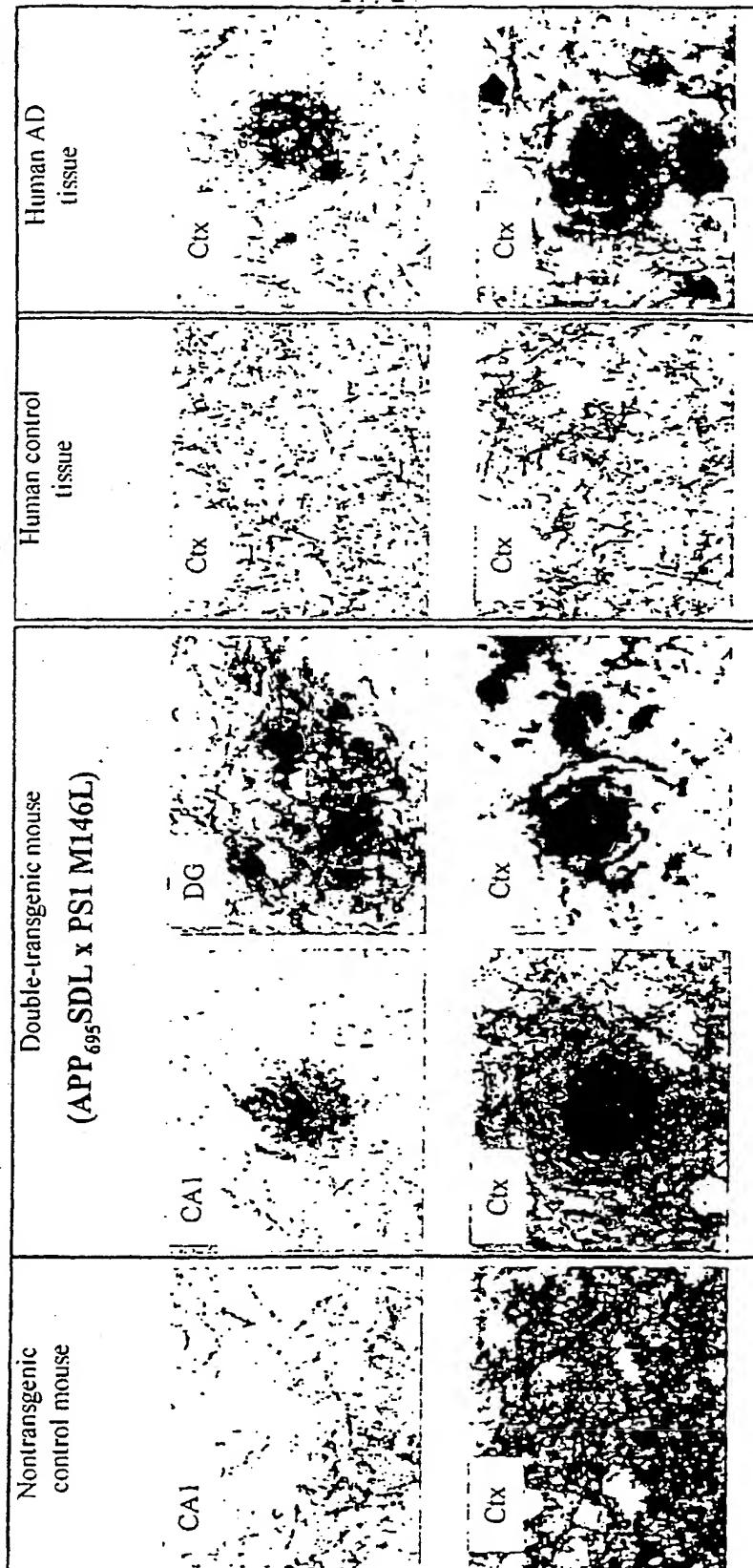
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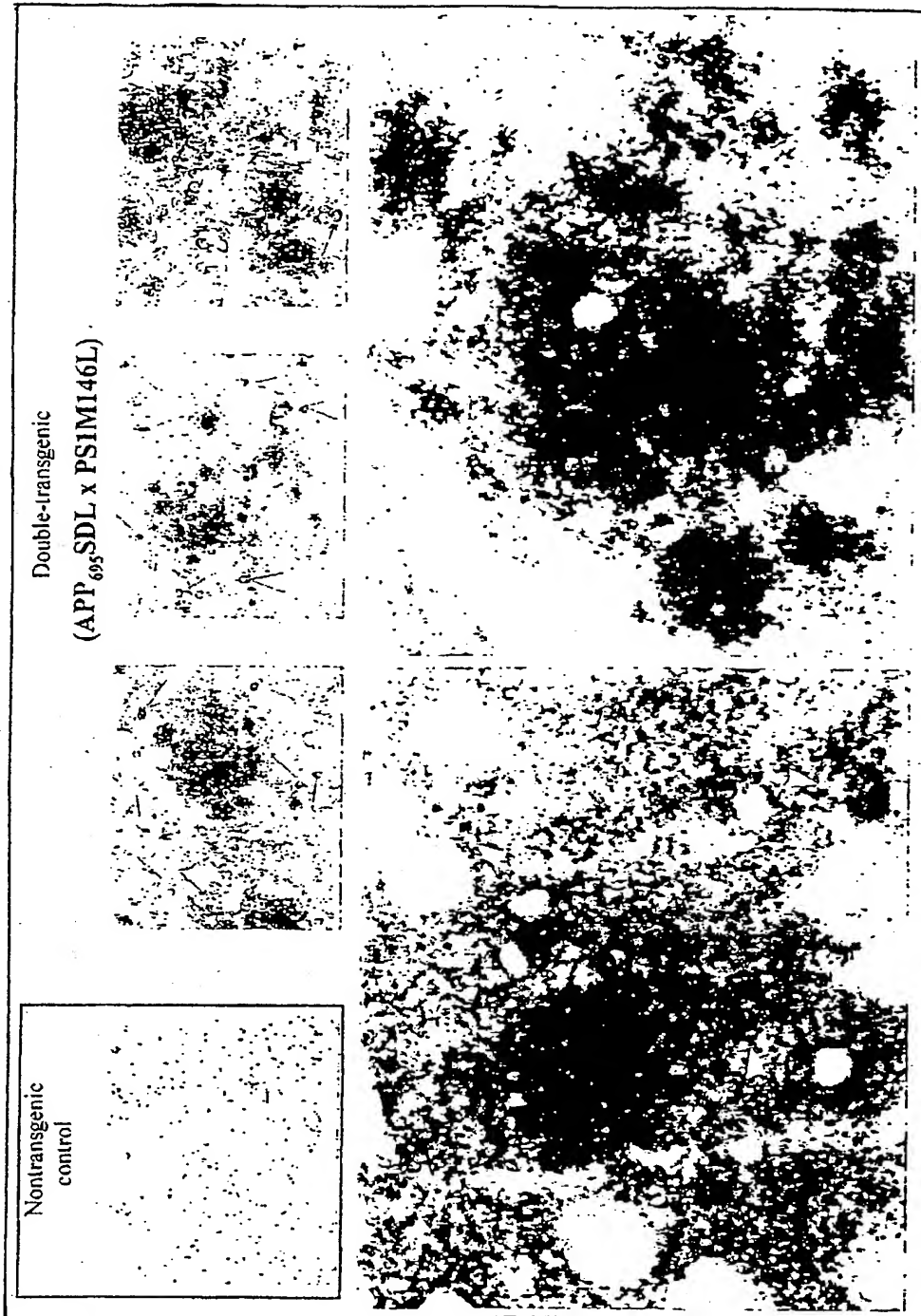
Figure 11B

Figure 12A

Figure 12B
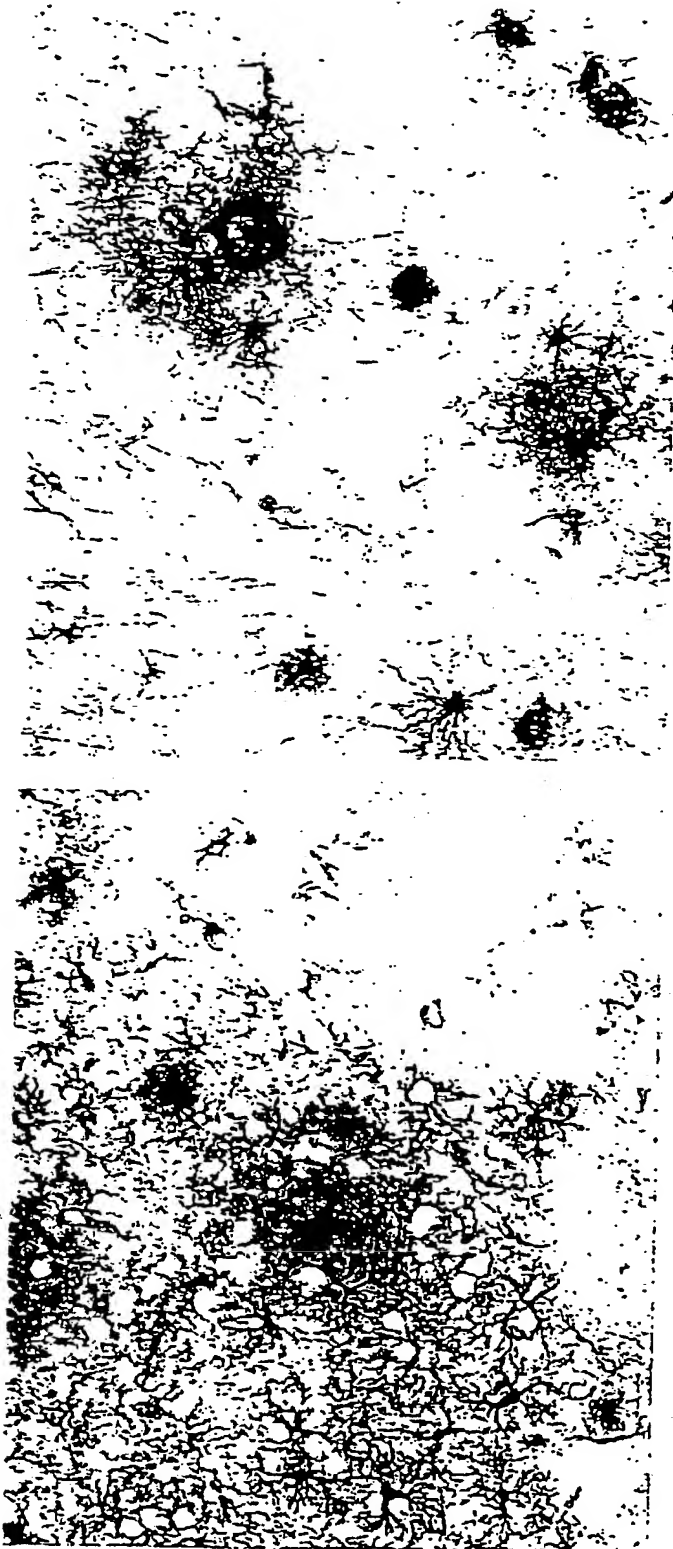
Figure 13

Figure 14B

Figure 14A

Figure 15

Figure 16

Figure 17B

Figure 17A
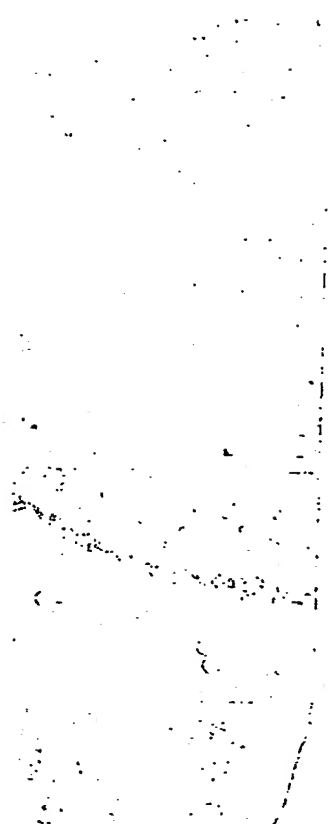
Figure 18A

Figue 18B

Figure 18C

Figure 18D

Figure 18E

Figure 18F

Figure 18G

Figure 18H
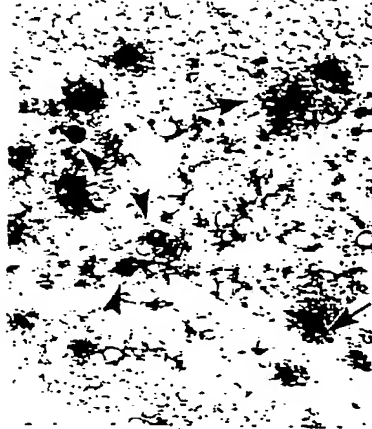
Figure 18K

Figure 18L

Figure 18I

Figure 18J
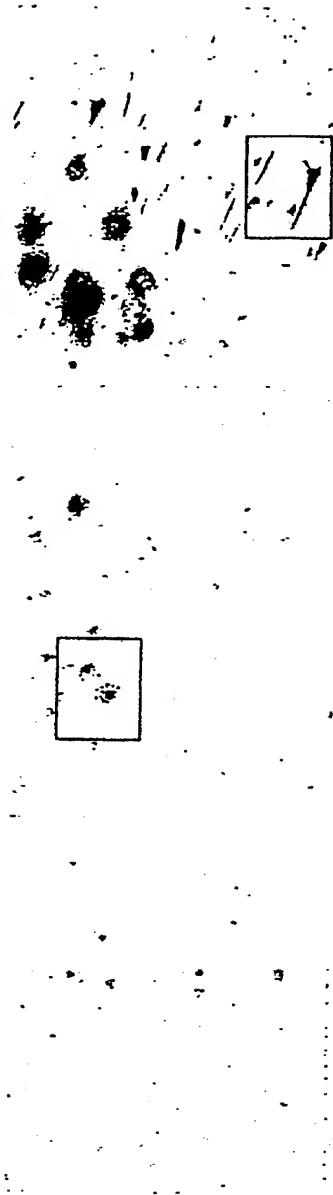
Figure 19A

Figure 19B

Figure 19C
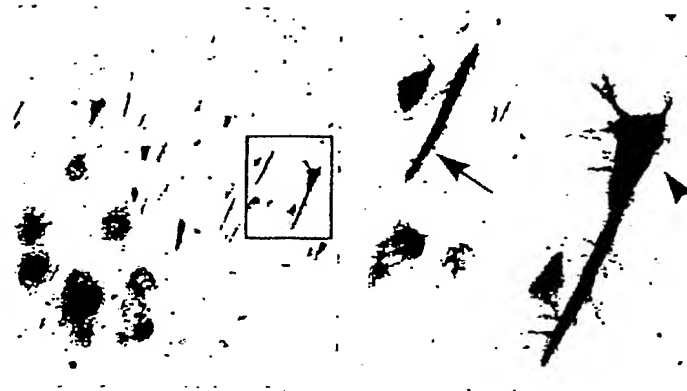
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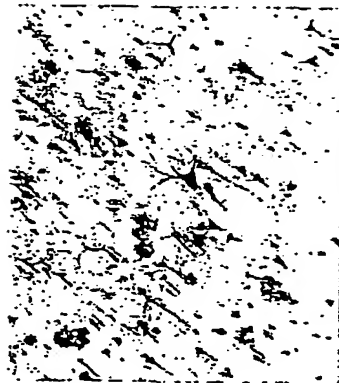
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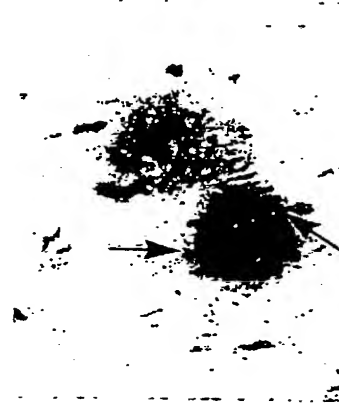
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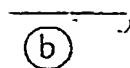
Figure 20A   Figure 20B
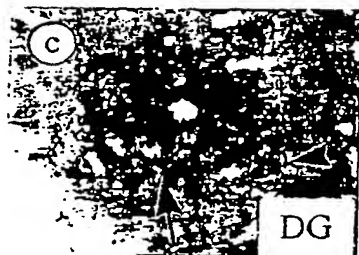
Figure 20C   Figure 20D

Figure 20E   Figure 20F

CA1
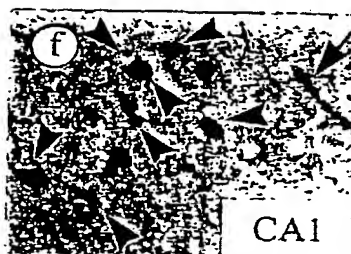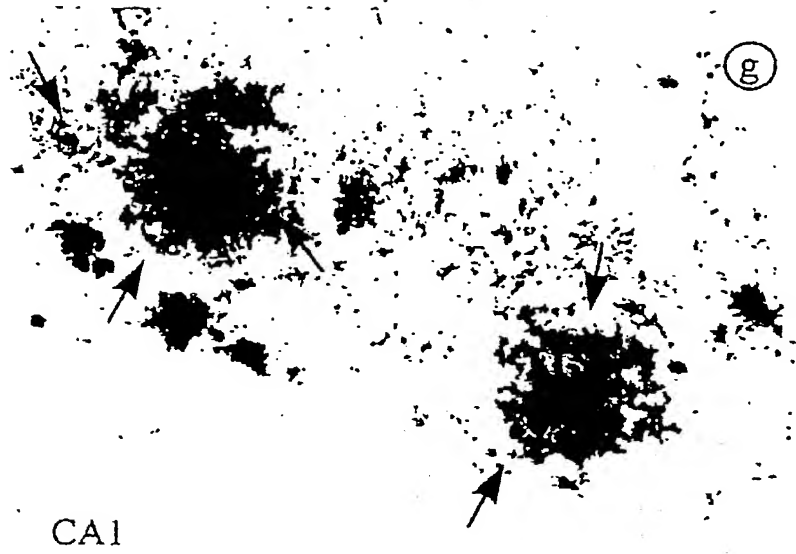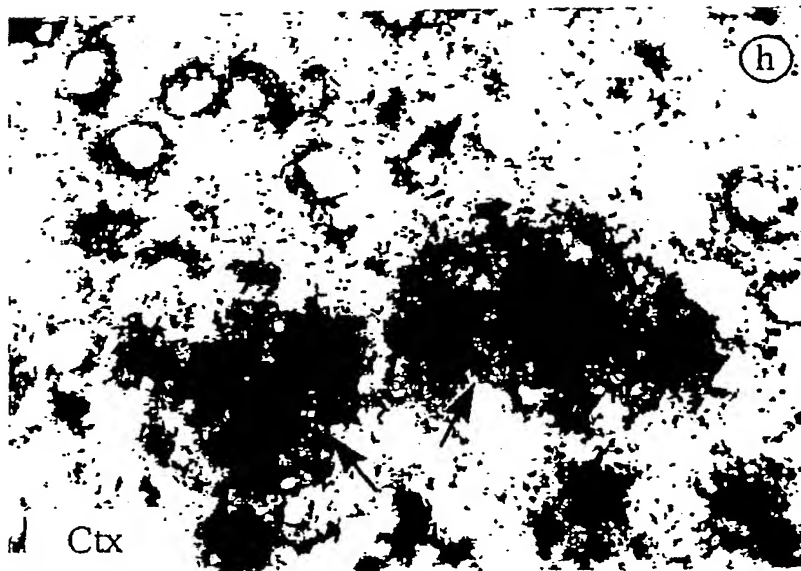
Figure 20G

Figure 20H

Figure 21A
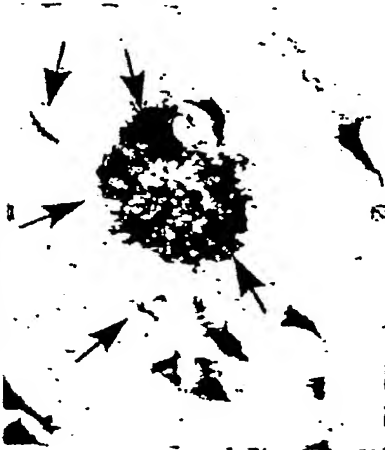
Figure 21B

Figure 21C

Figure 21D

Figure 21E

Figure 21F

Figure 22H

Figure 22J

Figure 22G

Figure 22I

Figure 22D

Figure 22E
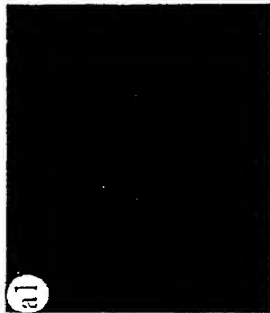
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Figure 22A
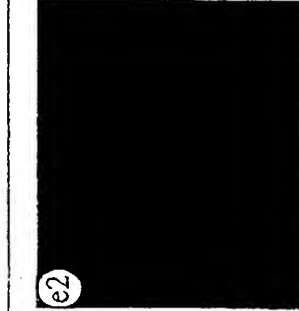
Figure 22B

Figure 22C

Figure 23A

Figure 23D

Figure 23B

Figure 23E

Figure 23C

Figure 23F

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☑ Application No. ___10/088,138_____, filed on___15 March 2002_____,

☐ as amended on _____(if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/ we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Saliha Moussaoui-Mrabet

Signature: _____ Citizen of: _Morocco_____

Inventor two: Veronique Blanchard-Bregeon

Signature: _____ Citizen of: _France_____

Inventor three: Assunta Imperato

Signature: _____ Citizen of: _Italy_____

Inventor four: Bruno Bonici

Signature: _____ Citizen of: _France_____

☑ Additional inventors are being named on ____I_____additional form(s) attached hereto.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

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All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Saliha Moussaoui-Mrabet _____

Signature: _____ Citizen of: Morocco _____

Inventor two: Veronique Blanchard-Bregeon _____

Signature: Veronique Blanchard 10/16/02 Citizen of: France _____

Inventor three: Assunta Imperato _____

Signature: _____ Citizen of: Italy _____

Inventor four: Bruno Bonici _____

Signature: Bruno Bonici 16/10/02 Citizen of: France _____

☑ Additional inventors are being named on ___1___ additional form(s) attached hereto.

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FULL NAME OF INVENTOR(S)

Inventor five: _Gunter Tremp_____

Signature: _____ Citizen of: _Germany_____

Inventor six: _Christian Czech_____

Signature: _____ Citizen of: _France_____

☐ Additional inventors are being named on _____additional form(s) attached hereto.

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FULL NAME OF INVENTOR(S)

Inventor five: __Gunter Tremp__

Signature: _____ Citizen of: __Germany____

Inventor six: __Christian Czech__

Signature: _10/14/02 Christian Czech_ Citizen of: _France_

☐ Additional inventors are being named on _____additional form(s) attached hereto.